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Reactive Heterocycles for Examining Polyketide Biosynthesis

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REACTIVE HETEROCYCLES FOR EXAMINING POLYKETIDE BIOSYNTHESIS

A Dissertation Presented

by

GITANJELI PRASAD

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DEDICATION

To my loving family and Chandru

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ABSTRACT

REACTIVE HETEROCYCLES FOR EXAMINING POLYKETIDE BIOSYNTHESIS

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Polyketides are a class of natural products that exhibit remarkable structural and functionally diversity and are highly sought after due to their medicinally important activities. For many decades now, polyketide synthases (PKSs), the mega-enzymes responsible for biosynthesis of polyketides have been the focus of extensive investigation to make new polyketides by polyketide engineering strategies. While there are many established methods to investigate polyketide enzymes and biosynthesis mechanisms, they have substantial shortcomings that have limited the extent of success with polyketide engineering efforts.

This thesis focuses on developing simple, flexible yet powerful tools for examining polyketide biosynthesis by overcoming some deficiencies in currently used techniques. Reactive heterocylces have been designed for direct labeling of key polyketide synthase enzymes to provide a direct insight into its functions and mechanisms. First β-lactones and then β-lactams have been used as small molecule probes to perform site-specific labeling of acyl carrier proteins and further used for mechanistic interrogation of key steps in polyketide biosynthesis. The utility of these probes has been demonstrated by comparison to traditional probes and has been successfully applied to examine substrate selectivity of keto synthases, key enzymes in polyketide biosynthesis. The applications of the tools described in this manuscript only scratch the surface of their capabilities and are expected to significantly aid in the study of new and

existing PKS systems leading to improved understanding of how these extraordinary biosynthetic machines function.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

CHAPTER 1

INTRODUCTION

1.1. Polyketides and Polyketide synthases

Polyketides are a diverse class of natural products found in soil and marine bacteria as well as fungi and higher plants. In addition to exhibiting a large range of structural and functional diversity, they are a rich source of biologically active and medicinally relevant compounds. Despite the vast structural diversity observed in polyketides, their biosynthesis proceeds by simple, repetitive condensations of acetate or propionate type monomers in a manner that closely resembles mechanism of fatty acid synthases (FASs) [1-3]. They are constructed by multi-enzyme assemblies termed as polyketide synthases (PKSs) that assemble simple acyl extender units in a linear fashion and yet this simple foundation is capable of producing molecules with enormous variation in structure and function. Polyketides can be broadly classified into groups based on how their parent polyketide synthases function. Iterative PKSs use the same set of active sites to build a polyketide chain and perform processing on them to form aromatized or highly oxidized products. The same module repeatedly catalyzes chain elongation and modification. In contrast, modular PKSs consist of sequentially linked groups of active sites called modules to perform chain elongation and other transformations to give more reduced products that often undergo macrocyclization.

1.1.1 Importance and Scope of PKSs

A large number of polyketides have been used as commercial pharmaceutical products and include numerous antibiotics (e.g. erythromycin, tetracycline), anticancer agents (e.g.

doxorubicin, epothilone B), immunosuppressants (rapamycin), antifungal (e.g. amphotericin), cardiovascular agents (e.g. lovastatin) and veterinary products(e.g. tylosin) (**Figure 1.**). Over the past few decades, primarily due to their importance as pharmaceutical agents, polyketides have been the focus of extensive investigation[1,2]. The prevailing problem of anti-bacterial resistance towards existing antibiotics has highlighted the need for new and effective therapies. There has been a troubling decline in emerging therapeutic leads in the pharmaceutical pipeline over the past few decades, resulting in increased demand for newer sustainable methods for drug discovery.

Despite significant success in synthetic drug development efforts, natural systems are still a rich source for potent small molecule therapeutics. Although polyketides are an attractive target for drug discovery, their structural complexity impedes their synthetic preparation. The total synthesis of such natural products is extremely difficult, laborious and suffers from poor yields. Synthetic modification of existing scaffolds has some promise but can be extremely labor intensive and is limited in the range of transformations that can be selectively performed on these complex natural products.

PKSs produce compounds with high medicinal activity and their structural and functional modularity has raised the possibility that polyketide biosynthesis pathways could be rationally reprogrammed by combinatorial manipulation to generate novel compounds of therapeutic significance.[7-13] Many aspects of polyketide engineering can be manipulated to achieve diversification. Product chain length variation can be achieved by varying the total number of modules in the PKS. Novel functionalities can be incorporated by introducing a variety of new primer and extender units, subject to the condition that substrate selection is controlled by AT enzymes. The oxidation state of the β-carbon as well as the stereochemistry can be modified by manipulating the DH, KR and ER of a module. Success in this direction is highly dependent on the

basic mechanistic understanding of the manner in which these molecules are biosynthesized [13] and significant efforts have been made to use PKSs as platforms to generate newer and diverse drugs and therapeutics.

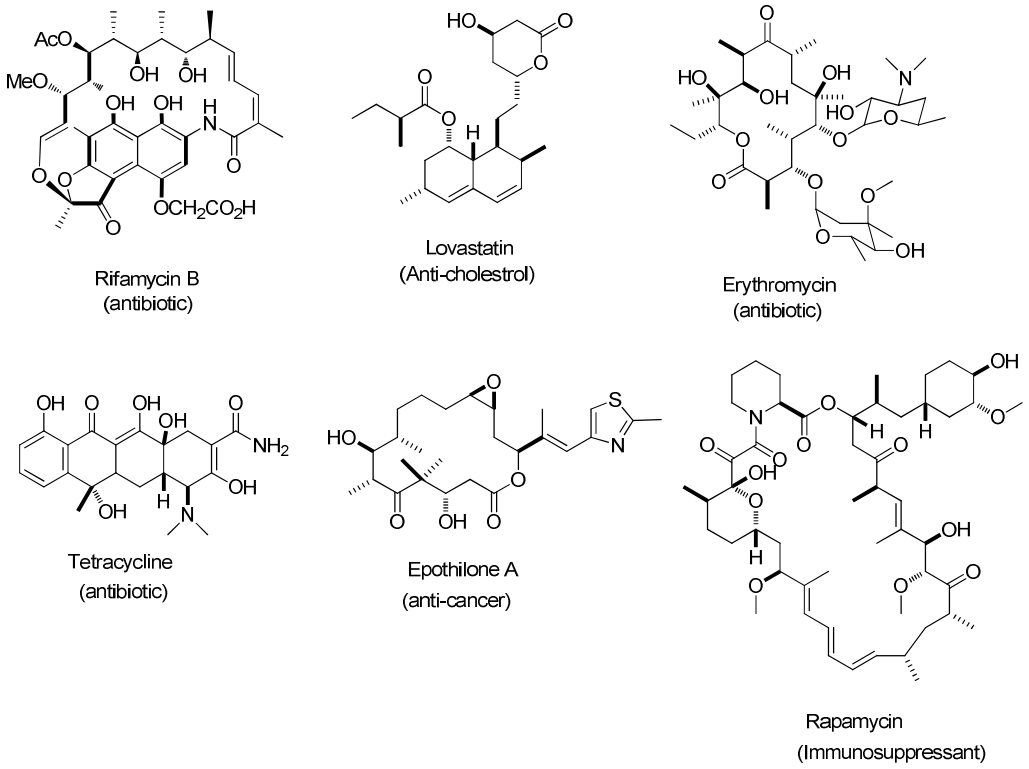

Figure 1. Examples of commercial pharmaceutical drugs

Particularly in modular PKSs, there exists a distinct linearity in the protein structural organization and their function. This makes them a powerful model for efforts towards design and engineering of polyketide biosynthesis. Extensive work has been done with the aim of mixing and matching modules and domains from heterologous PKS proteins. Another approach is to use domain deletion, addition and domain swapping to generate novel, ''unnatural'' polyketides. Numerous studies have been aimed to better our understanding of natural systems and in turn, improve our ability to manipulate them [14-17]. However, these efforts have only

achieved moderate success and are often faced with the issue of decreased productivity which could be due to structural instability of the engineered protein, sub-optimal chemistry within the altered module or problems with inter-modular transfer of polyketide intermediates in the assembly line [14-17]. Another major hurdle is the intrinsic specificities within key enzymes responsible for production of polyketides which arises from lack of understanding their natural preferences. Therefore there is an urgent need to explore more reliable means of investigating the molecular basis for key aspects of substrate selectivity of enzymes, intermodular communication and intermodular chain transfer.

The research described in this thesis is aimed towards developing a set of highly desired molecular tools to facilitate the study of polyketide biosynthesis and ultimately improve polyketide engineering.

1.2. Modular Polyketide Construction

Modular PKSs are assemblies of covalently connected catalytic domains in the form of a large polypeptide. Each domain catalyzes a single chemical transformation and are collectively organized into modules. Each module, composed of its set of domains, brings about one round

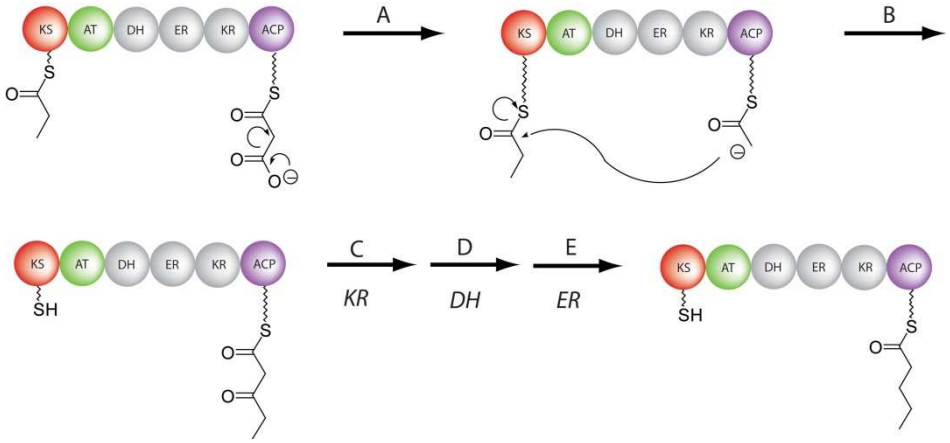

Figure 2. Proposed mechanism for polyketide formation in modular PKS

of chain elongation to the polyketide backbone (**Figure.2**). The variety in the organization of these large protein assembly lines gives rise to polyketides of great structural diversity.

Chain growth is initiated by a loading module, followed by a number of extender modules that make up the body of the polyketide chain and the process is terminated by a release module. The first module contains a loading domain often composed of a loading acyl transferase (AT) domain and a loading acyl carrier protein (ACP) which loads the first extender module with an acyl substrate. Each extender module must contain at the least, an AT, ACP and ketosynthase (KS) domain to function. In addition to this, they may also contain combination of β-carbon processing enzymes such as a ketoredcutase (KR) domain, a dehydratase DH) domain, and an enoylreductase (ER) domain. The composition of these domains varies widely across different PKSs. The final module in this sequence generally contains a C-terminal thioesterase (TE) domain which releases the final polyketide product from the PKS [4-7].

Polyketide synthesis proceeds in a manner that resembles an assembly line with individual components performing distinct tasks in a stepwise fashion to a specific product repeatedly. A starting unit in the form of a Coenzyme A derivative such as Propionyl-CoA is loaded onto the terminal thiol of the phosphopantetheinyl arm of the ACP of loading domain by the AT of the loading domain to form a thioester-bound acyl fragment. Chain extension begins with passing of the acyl fragment from the ACP to the cysteine active site of the first extender module KS (**Figure 2**). The AT loads the ACP of this module with an appropriate malonate-derived extender unit. The AT strictly controls the choice of extender unit for a particular module and hence acts as gate keeper. The KS of this extender module catalyzes a decarboxylative Claisen condensation between its acyl unit and the extender unit on ACP forming an ACP-bound β-ketothioester product. The β-keto residue may then undergo sequential processing by KR to from an alcohol, DH to form an alkene and ER to form a

saturated chain. Depending on the module, a given elongation step may stop at any of these intermediate products (keto, hydroxyl, enoyl) or result in the fully reduced β-carbon product. Upon completion of these steps, the chain is passed from ACP of this module to KS of next downstream module and the sequence of growth and processing repeats until the desired polyketide product is formed. Once the product is completed, the chain is passed to the TE where it catalyzes release of the product either by hydrolysis or cyclization. The choices of building blocks made by each module and the number and types of domains within each module catalyzing postcondensation reactions dictate the chemical functionality at each carbon atom in the final product.

1.2.1. 6-derythronolide B synthase (6-DEBS)

The most well studied modular PKS is 6-deoxyrythronolide B synthase (DEBS) which is responsible for assembly of 6-deoxyerithronolide B (6-DEB), the macrolide precursor to

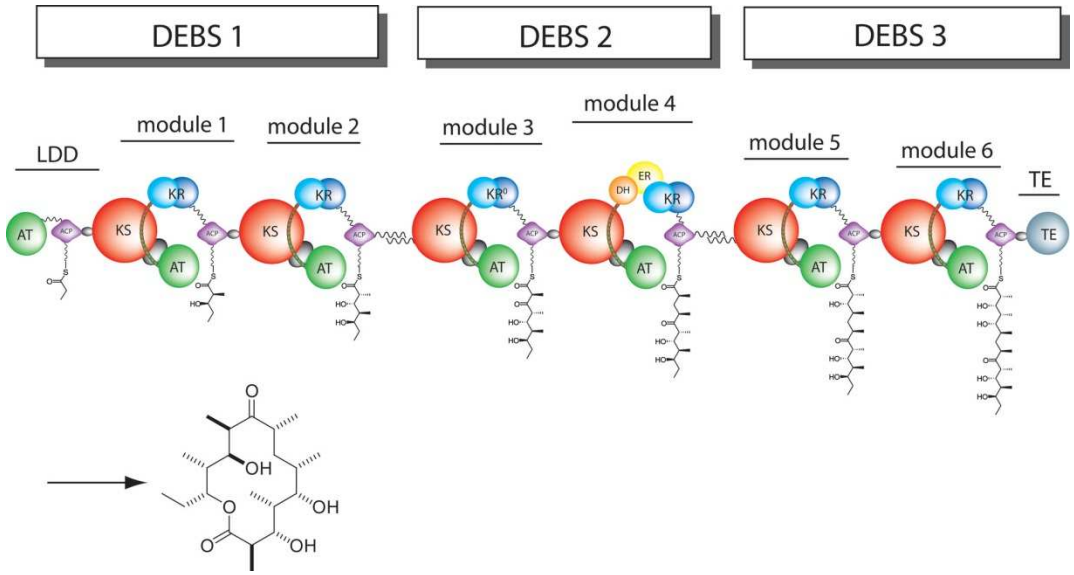

Figure 3. 6-deoxyerythronolide B synthase (DEBS)

erythromycin A. 6-DEBS has become the archetype of modular PKSs. The entire DEBS has been successfully expressed in the heterologous hosts such as *Escherichia coli*. Recombinant strains of *E.coli* have been engineered specifically to optimize the expression of the DEBS proteins[4-6]. The ability to efficiently express PKS proteins in engineered heterologous hosts has provided a greatly simplified platform to perform PKS investigations.

DEBS consists of six modules grouped into 3 large polypeptides DEBS1, DEBS2 and DEBS3 (**Figure 3**.) where each contains two modules. The loading didomain consisting of ACP_L and AT_L, loads propionyl-CoA onto the KS of the first extender module. The AT of the first module loads the ACP of that module with methylmalonyl-CoA as an extender unit. Similarly, all other ATs in DEBS incorporate a methylmalonyl-CoA unit onto their respective ACP. All six modules harbor a KR, while KR of module 3 is inactive. Module 4 carries a DH domain and ER domain as well as a TE at the C-terminus for releasing the final macrocycle. As in all PKSs, the precise composition and specificity of these domains provide the diverse stereochemistry and oxygenation patterns in the final macromolecule. Much of what is known about the molecular mechanisms of modular PKSs is derived from extensive investigations of the DEBS. These large PKS modules were not very suitable for performing in-depth investigation of mechanistic steps of each individual domain. A substantial advancement in this area came from limited proteolysis studies that revealed highly conserved interdomain boundaries at which individual modules could be further disconnected to form fully functional PKS subunits. Upon proteolytic cleavage, functional domains are released and their exact domain boundaries are calculated by N-terminal sequencing analysis [12,13]. This gives rise to domains such as KS-AT, ACP, TE, AT-ACP loading didomain and other beta carbon processing sub-units as stand-alone entities. For instance, by identifying cleavage sights located in the amino acid sequence, it was possible to express a homodimeric [KS][AT] didomain possessing full catalytic activity that could be used for stepwise

structural and functional analysis [13]. This approach has been used extensively to perform in-depth analysis of DEBS enzymes and their functions which in turn have helped understand the working modular PKSs.

1.3. Traditional Methods for PKS investigations

In the past, a number of approaches have been utilized to investigate various aspects of the PKS biosynthetic machinery. Some key approaches have been described in the following sections. Despite having their uses in some areas of application, they suffer from certain shortcomings in other areas which reinforce the need for newer tools for investigation of PKSs.

1.3.1. Radiolabeling

For many decades now, radiolabeling has been extensively used for *in vivo* as well as *in vitro* PKS biosynthesis investigations. DEBS is the most well studied PKS using these techniques. Traditionally, it was used for incorporation *in vivo* of radiolabelled substrates to probe the mechanism of biosynthesis by analyzing of the final product[18a, b]. The radiolabelled substrates, [2-$^{2}H_2$, 2-^{13}C] propionate and [2-$^{2}H_2$, 2-^{13}C] methylmalonyl-CoA were fed to the bacterial cultures of *S. Erythrea*, and the polyketide product were isolated and analyzed by scintillation count and using ^{13}C NMR. The incorporation of the radiolabels into the erythromycin macrolide gave insight about the mechanism of condensation and chain extension. The observation of specific stereochemistry such as generation of *D*-methyl groups in the carbon skeleton of the final molecule provided strong evidence for analogy to fatty acid biosynthesis. However the scope of this approach limited due to poor yields *in vivo,* inability to isolate crucial intermediates and failure to address key aspects such as protein-protein and protein-substrate interactions,

substrate recognition of individual domains and precise mechanism of individual steps in chain transfer and elongation.

1.3.1.1 Radiolabeling with 6-DEBS

A more direct way of gaining insight into these key questions involved carrying out experiments with PKS proteins *in vitro* which made it possible to probe the PKS multienzymes under controlled conditions such as temperature, buffer, and in the absence of metabolic constraints. The use of heterologous hosts such *E. Coli* to express these proteins in good yields has greatly facilitated this approach. Additionally, *in vitro* environments provide a cleaner background for the isolation of polyketide products. To facilitate this approach, the DEBS was shortened into smaller model systems by moving the thioesterase (TE) domain from DEBS3 to the end of DEBS1 and DEBS2 to form DEBS1+TE and DEBS2+TE resulting in premature release of the chain at the triketide stage [20]. This revolutionary approach introduced an efficient alternative to working with these enormous intact protein systems. The truncated modules were incubated with radiolabelled coenzyme A derivatives to obtain chain extension and release of the cyclized products which were analyzed by thin layer chromatography with autoradiography or phosphorimaging. For example, incubation of module 2+TE with appropriate analogue of the natural diketide-SACP substrate of KS of DEBS module 2 and MeMal-CoA for the ACP gives an acyclic triketide that is released from the enzyme by TE catalyzed cyclization to yield triketide lactone (**Figure 4.**). The radiolabeled product were extracted out of the samples and analyzed by liquid scintillation counting which quantifies the amount of radiolabeled product formed. The amount of radiolabeled product provides measure of enzyme loading and substrate turnover.

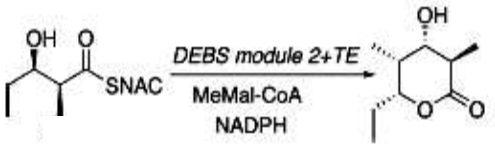

Figure 4. Conversion of Diketide-SNAc to Triketide lactone

The use of isolated PKS enzymes has made it possible to perform extensive biochemical analysis of substrate specificity, kinetics, and stereochemical preferences [14-17, 20]. However, an intrinsic limitation of *in vitro* studies is the greatly reduced (~1µg) quantities of polyketide products that are ordinarily generated in such experiments, which require the independent preparation of authentic reference samples by laborious total synthesis. Additionally, the high cost of required coenzyme A thioesters needed for chain extension prohibits larger scale *in vitro* synthesis of polyketides.

As described previously, limited proteolysis studies yielded all the individual domains from DEBS. Isotopically labeled SNAc thioesters are extensively used along the same lines as described above with these stand-alone protein units by loading the ACP and KS domains and performing chain elongation [21]. By far, the most significant advantage of this method is the ability to carry out multiple turnover experiments with discrete ACP and KS-AT domains in the absence of TE which was previously necessary to release covalently bound products from the enzyme.

1.3.1.2. N-acetylcysteamine thioesters

The discovery of simple synthetic mimics of coenzyme A substrates in the form of N-acetylcysteamine(SNAC) thioesters brought about a great advancement in this field. These SNAC thioesters were more economical, easily prepared and demonstrated to be good substrates of PKS enzymes [22]. The N-acetylcysteamine (NAC) is a truncation of the coenzyme A side chain and

hence acts as the recognition motif for PKS enzymes. In the first report, the methylmalonyl-SNAc was incubated with DEBS3 along with a [^{13}C] labeled diketide primer to test product formation. The organic extract of the reaction was analyzed by ^{13}C NMR and confirmed the formation of the expected triketide lactone. This experiment confirmed the acceptance of these SNAc thioesters as good substrates for PKS enzymes, following which, a large number of other SNAc thioesters have been prepared and widely used as coenzyme A substitutes.

As an example of radiolabeling experiments of DEBS hybrid modules with use of SNAc

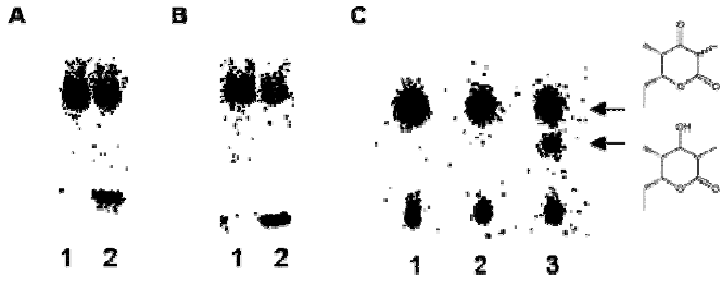

Figure 5. Analysis of radiolabeling experiments

(A): Radio SDS−PAGE of [^{14}C] 2-methyl-3-hydroxylpentynoyl-SNAc labeling of M3M5TE hybrid module and ACP2. (B): Radio SDS−PAGE of [^{14}C]Methylmalonyl-CoA labeling of M3M5TE and ACP5 and (C): Radio TLC Triketide lactone formation by DEBS hybrid module. [21]

thioesters and their analysis, **Figure.5** outlines an assay performed with hybrid module containing ACP3, KS5 and TE of DEBS (M3M5TE). The module is incubated with substrates for ACP and KS and the labeled proteins are visualized by radio-SDS-PAGE whereas the small molecule output of triketide lactone is visualized by radio-TLC.

In all, radiolabelling techniques have been vastly used to probe the activities and behavior of various PKS enzymes. The major drawback of this approach is the prohibitively expensive reagents that limits the availability of diverse substrates and restricts applications to

smaller scales. Here lies the gap in biotechnology to develop simpler strategies to investigate PKS enzymes and biosynthesis which can overcome the handicaps discussed above.

1.3.2. Mass spectrometry

More recently, mass spectrometry has emerged as a powerful tool for biological analysis as a result of its sensitivity, accuracy and speed and has been used for structure elucidation of polyketides and observing aspects of PKS biosynthesis [29-34] During the biosynthesis of polyketides, substrates, intermediates and side products are covalently tethered to the PKS, introducing mass changes, making these biosynthetic systems ideal candidates for interrogation by large molecule mass spectrometry. MS can be used to understand substrate tolerance, timing of covalent linkages, timing of tailoring reactions and the transfer of substrates and biosynthetic intermediates from domain to domain.

Controlled limited proteolysis has been used to release and separate entire functional domains from modular PKSs such as DEBS which enables to probe the function and organization of the domains within the intact modules [12,13]. For instance, DEBS hybrid units such as DEBS1-TE, DEBS3 and the DKS(loading didomain+module1, called diketide synthase) were subjected to this method [31]. The intact muti-domain units were digested with trypsin and monitored by LC-MS to separate the fragments and analyze them using sequence alignments and N-terminus sequencing to identify the separated domains. For instance, the DEBS1(loading didomain+module1+module2) proteolysis gave rise to many single and multidomains such as KS1, AT1, TE, KR1, ACP2-TE, AT_L-ACP_L(AT and ACP of loading domain). Similarly, the proteolysis of the DKS unit gave another array of domains. The ability to isolate these individual domain and domain pairs enabled the study of acylation specificities for each individual AT, ACP and KS. For

instance, Propionyl-CoA, the native substrate for the DEBS loading module, was incubated with intact DKS followed by proteolysis to release the domains for analysis. By analysis of mass peaks, it was revealed that propionyl units were specifically loaded onto AT_L-ACP_L and KS1 but not on AT1, KR1, ACP1 or TE domains. This indicated that propionyl-CoA is not a suitable substrate for the extender AT1 and ACP1 domain. Experiments with the non-natural substrate n-butyryl-CoA with DEBS1 and DKS showed similar results also indicating sufficient mass accuracy to distinguish between propionyl and butyryl groups. In this manner, numerous substrates studies have been conducted with these domains by observing the stable acyl intermediates.

Along similar lines, individual domains such as ACP, TE and [KS][AT] didomians were engineered and expressed in *E.Coli* [13]. A method was developed to achieve complete proteolysis of didomain to enable the direct detection of covalently bound substrates by MS. For example, in an experiment, the substrate specificity of KS from DEBS module 3 was examined by pairwise combinations of various di- and tri-ketide-SNAc thioesters and observation of KS acylation. The specificity for each substrate was measured by the relative proportions of each acyl-enzyme species, as determined by LC-MS (**Figure 6**.). Also, by doing time points of incubation, relative rates of acylation and step-wise kinetic analysis can be performed. KS-catalyzed chain elongation in the presence of the methylmalonyl-ACP was performed with these different KS substrates. The use of discrete ACP and [KS][AT] domains allowed multiple turnover experiments by use of excess ACP. Direct proteolysis of the mixture followed by LC-MS analysis provided peptide fragment peaks corresponding to the KS and ACP domains at various stages of polyketide extension. Kinetic analysis provides detail insight into comparative rates of ACP loading, KS acylation by diketide substrate and chain elongation event. One major advantage of this method over traditional radiolabeling is that both acylation and elongation can be

monitored simultaneously. It has also eliminated the need to use expensive radiolabels for PKS examinations.

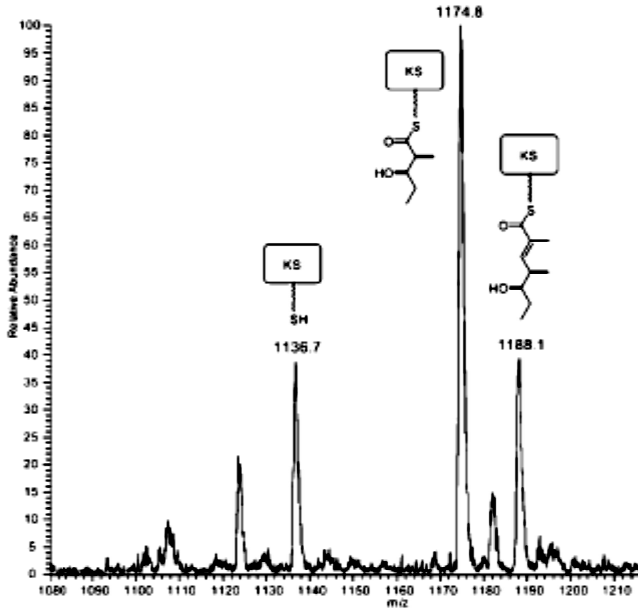

Figure 6. [KS][AT] didomain acylation observed by LC-MS

In this manner, mass spectrometry has grown into powerful yet sensitive and reliable method to assay ACP and KS acylation events. It has been used in limitless application including study of substrate specificities of PKS enzymes, examination of various heterologous ACP-KS pairs, kinetic evaluation of chain elongation steps and PKS protein chemistry.

Despite many improvements over traditional techniques, mass spectrometry has some shortcomings. Firstly, the full PKS modules that are about 300 kDa in size cannot be used in this assay due to which smaller subunits are used as substitutes such as the [KS][AT] didomain. It remains unclear whether such a substitution has any effect on actual PKS enzyme specificities. Secondly, there is the question of effect of substrates on ionization potentials when proteolysis is performed. This could adversely affect data interpretation and its reproducibility. Lastly and

most importantly, substrates with high hydrophobicity are susceptible to solubility issues which affect the signal in MS which limits the chemical diversity of substrates.

1.4. Hypothesis

As summarized in this chapter, mass spectrometry and radiolabeling strategies combined with genetic engineering and molecular biology tools, have been successful for many aspects of PKS investigations. The high reagent cost, limited substrate availability in case of radiolabeling, and inability to perform direct PKS investigations using MS methods restrict their

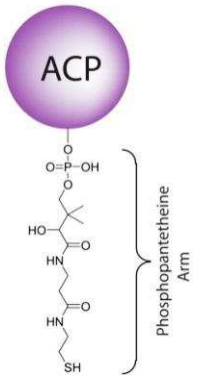

Figure 7. *holo*-Acyl Carrier protein with Ppant arm

use in medium to high-throughput applications essential for investigating the rapidly emerging new PKS systems. There exist many reliable methods to study KS substrate specificities by directly loading the KS active-site which have been discussed above. This has enabled enormous KS-centered analysis of chain elongation kinetics, inter-domain and inter-modular chain transfer but little is known about other aspects, for example, the tolerance of KS towards diverse non-natural extender units.

Nearly all biosynthetic steps associated with modular PKS systems rely on acyl-ACPs. Therefore, functional analysis of each enzymatic event would require a robust method of

generating and monitoring their fate. Despite the central and highly functional role played by ACPs in polyketide biosynthetic processes, ACP-based PKS examination strategies are highly underdeveloped mainly due to insufficient techniques to perform ACP modifications. The strict AT specificities limit the variety of natural substrates for ACP and have prevented efforts of AT-mediated modification of ACP using synthetic substrates. Traditionally, ACPs are modified by small molecules via acylation of coenzyme A and subsequent PPant transferase-catalyzed transfer to the *apo* form of ACP. Synthesis of these CoA analogues is labor intensive, costly, and scale-up is tricky[23-26]. Another method utilizes the CoA pathway to introduce thioether-linked substrates onto the ACP via generation of synthetic CoA analogues [27-29]. While a very powerful approach, this technique requires extensive genetic manipulation and resulting thioesters cannot be processed by PKS enzymes that recognize thioester linked species and therefore are of limited use to PKS investigations. As genomic-based technologies continue to supply an abundance of new PKS systems, there is an urgent need for complimentary methods using simple, reliable and structurally flexible ACP-acylating agents.

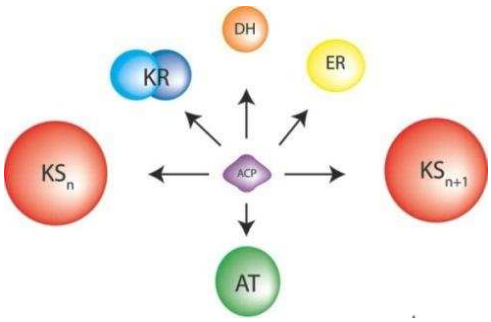

Figure 8. Acyl Carrier Protein enzyme partners

We were convinced that ACPs, with their ability to interact with many proteins and carry out a range of vital functions, were the ideal choice for introducing chemical substrates, labels, and intermediates onto PKSs (**Figure 7, 8**). To achieve this, there is a need to develop a method

to introduce novel substrates with wide structural diversity onto the ACP and generate functional donor-ACPs to utilize them in PKS investigations. We reasoned that a suitable strategy would be to design appropriately reactive small molecule functionalities and target them towards a biomacromolecular site within the PKS. The terminal thiol PPant of ACP is a unique nucleophile in PKSs and being positioned on a long flexible prosthetic arm imparts it great accessibility and distinctive reactivity to ACPs in the presence of a multitude of other nucleophiles such as cysteine, serine, lysine and tyrosine.

As outlined in the rest this document, our aim is to target the PPant arm of the acyl carrier protein. The hypothesis is that, with the right electrophilic partner for this terminal thiol using small molecules, we can achieve site-specific labeling and generate acyl-ACPs to gain a direct access into the PKS machinery.

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CHAPTER 2

DIRECT ACYLATION OF CARRIER PROTEINS WITH β-LACTONES

2.1 Introduction

Acyl carrier proteins are critical components of fatty acid and polyketide biosynthesis . Their primary function is to shuttle intermediates between active sites *via* a covalently bound phosphopantetheine arm. Majority of biosynthetic steps involved in modular PKS systems rely on acyl-ACPs [3]. Therefore, a reliable method for generating acyl-ACPs and monitoring their fate, will greatly aid step-wise functional analysis of PKSs. Our hypothesis was that the Ppant group may be uniquely reactive, given an appropriate electrophilic partner, and therefore, targetable for direct acylation. We reasoned that small molecules capable of acylating this prosthetic group will provide a simple and reversible means of introducing novel functionality onto carrier protein domains. We were interested in using β-lactones for directly labeling acyl carrier proteins (ACPs) via the phosphopantetheine(Ppant). This strategy was particularly appealing since, β-Hydroxythioesters, formed by addition of the PPant thiol to a β-lactone, are common components of polyketide intermediates.

There is substantial precedence of electrophilic groups used as partners for nucleophiles in biological systems. Many biologically active natural products have electrophilic functional group cores that target nucleophilic residues as a part of their mechanism of action. Some well documented examples of such a feature are lipstatin, penicillin, cerulenin, orlistat [1,2]. They are perfect examples of how electrophilic groups such as the carbonyl group, the epoxide and the electron deficient alkene are protein-reactive chemical entities. These electrophiles covalently modify their nucleophilc targets to inhibit or deactivate them. The lipstatin family of natural

products features a four-membered β-lactone ring. The chemical reactivity of this ring is the basis of their biological activity. The catalytic active-site serine nucleophile of their target enzyme attacks the electrophilic carbonyl of the β-lactone to form a β-hydroxy serine ester. In case of lipstatin, it has been experimentally confirmed that the reaction is exclusively at the carbonyl site giving an ester and not an ether product[3].

2.1 Results and discussion

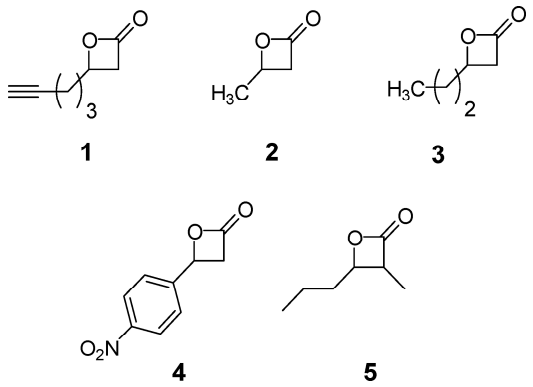

Figure 9. β-lactone for ACP-acylation

We began by preparing an alkyne-bearing β-lactone, compound **1** (**Figure 9.**)[5], for performing the ACP-acylation approach. The ACPs used for this study were from modules 2 (ACP2) and 3 (ACP3) of the 6-deoxyerythronolide B synthase (DEBS) that were isolated, overexpressed and purified in both *apo* and *holo* forms, using BL21 and BAP1 cells, respectively. To test selective Ppant acylation, the reagents were incubated with these isolated *holo*- and *apo*-ACP domains. The criteria for a successful reagent were to exhibit selectivity for the *holo* structures, without nonspecific acylation of the *apo* ones. Competition from nucleophiles present on other common PKS domains was a primary aspect to be observed carefully. We were aware that, to be truly useful, our probes must avoid reaction with them. Prior studies report KS

active site to be competent nucleophiles for β-lactone ring-opening [6], as a result, KS-AT didomain from DEBS module 6 (KS-AT6) was overexpressed and purified [7-8].

There is precedence in this matter, where minimal reactivity between compound **1** and the KS domain of bacterial fatty acid synthase was observed [6]. We were interested in testing the purified DEBS KS domain in this respect to see how it behaved in these conditions. In this manner, the stage was set to examine both efficiency and selectivity of ACP-acylation with β-lactones.

As discussed earlier, tandem proteolysis-mass spectrometry has emerged as a powerful means of detecting PKS-bound small molecules in vitro [9-11]. By extensive trypsinolysis of *apo*- and *holo*-ACPs, a series of peptide fragments were produced, including one containing the conserved DSL motif, to which the Ppant is attached in samples derived from BAP1 as determined by LC-MS. Incubation of *holo*-ACP2 and -3 with varied equivalents of **1** at pH 7 for 1 h followed by exhaustive trypsinolysis and LC-MS, to determine the fraction acylated, produced saturation curves which indicated that a roughly 50-fold excess of lactone is required for

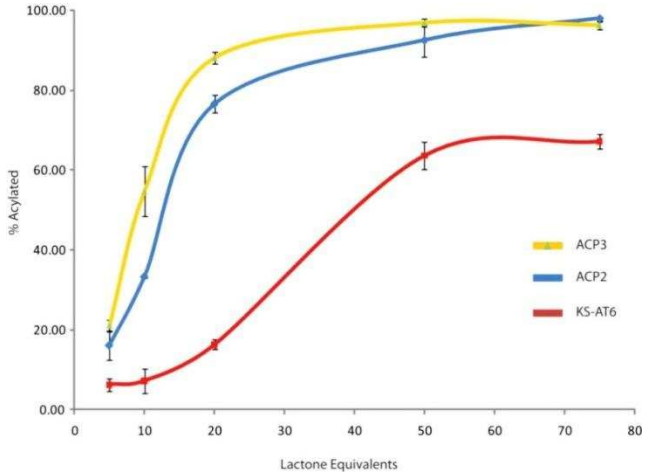

Figure 10. Saturation curves of alkyne β-lactone

ACP3 (yellow), ACP2 (blue), and KS-AT6 (red) treated with compound **1,** trypsinized and analyzed by tandem proteolysis-mass spectrometry. Equivalents of lactone are per protein molecule. Each data point is the average of three experiments. Standard deviations are shown as black error bars. Lines are added for clarity.

complete acylation (**Figure 10.**). The results were that both *apo*-ACPs showed no acylation with 50 equiv of lactone, providing further evidence that the reaction is occurring on the Ppant arm as this is the only difference between the *apo* and *holo* forms of ACP. In comparison, the saturation curve for KS-AT6 showed 25−50% less acylation than ACP for the range of lactone equivalents tested. Most importantly, at 10 lactone equivalents, the ACPs were approximately 35−50% loaded while the KS was mostly unreactive after 1 h of incubation. Standard deviations for these experiments were generally within 1−4% suggesting that the differential reactivity observed was genuine.

By means of tandem proteolysis/LC-MS it was confirmed that the acylation occurs at the PPant group. However, not all proteolysis products readily ionize under the conditions used. In addition, we could not rule out the potential for acylation of the peptide fragments over the intact protein. As an alternative, a gel-based assay was designed according to previous work by Sieber and co-workers [6]. Each ACP (*apo* and *holo*) and KS-AT6 was incubated for 1 h with 10 e SDS-PAGE analysis of the resulting mixtures showed bright fluorescent bands corresponding to *holo*-ACP2 and -3 while the *apo*-ACPs and KS-AT6 displayed only background fluorescence (**Figure.11.**). These results provide initial indication that β-lactones can be selective for ACP over KS as well as avoid other potential competing residues such as surface cysteines, serines, and lysines.quiv of **1** followed by 1,3-dipolar cycloaddition ("click") reaction with rhodamine azide [12-14].

At this point, the functionality of the acyl-ACP intermediates needed to be established along with the evidence of thioester over thioether formation. Preloaded ACP2 and ACP3 (10× compound **1**) were mixed with KS-AT6. After 1 h, the click reaction was performed as before and the proteins separated by gel electrophoresis.

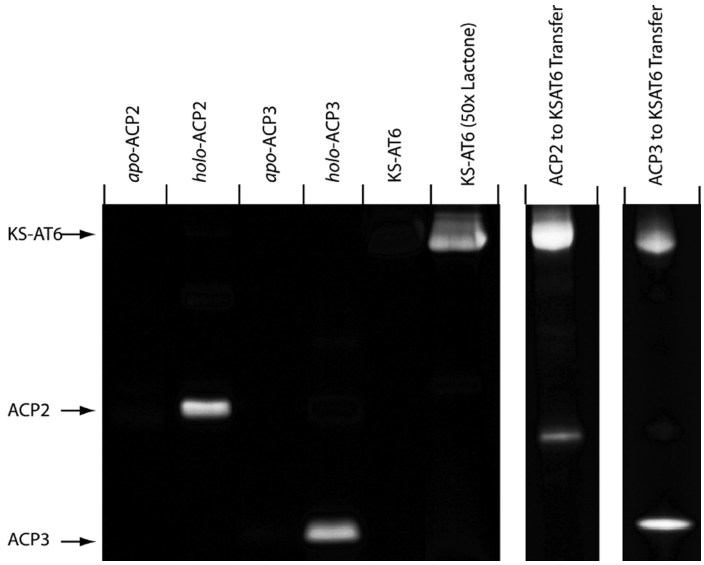

Figure 11. PAGE analysis of fluorescently labeled DEBS ACP3, ACP2, KS-AT3, and KS-AT6

Reaction with compound **1** and subsequent click reaction with rhodamine-azide. Lanes are marked above with the corresponding protein component. Markers to the left indicate expected bands for the indicated species. The right two lanes depict transfer of the acylation product from preloaded ACP to KSAT indicating that the intermediates are functional. β-Lactone was applied at 10× relative to protein unless otherwise noted. ACP2 contains a C-terminal linker region accounting for its larger mass.

Bright bands corresponding to the KS-AT6 didomain indicate that the β-hydroxythioester is efficiently transferred from the ACPs (**Figure 11**). It should be noted that direct KS-acylation is not observed under the conditions used in these experiments.

The next step was to examine β-lactone behavior with a higher level of biological complexity for which we overexpressed and purified *apo* and *holo* forms of the intact module 2 from spinosyn synthase (SpnB). This particular module was chosen because it contains KS, AT, DH, ER, KR, and ACP domains which represent the vast majority of synthase components present in a given assembly. Both *apo* and *holo* forms of spinosyn module 2 were incubated for 1 h with 10, 50, and 75 equiv of **1** followed by "click" reaction with rhodamine azide and PAGE analysis as before (**Figure 12.**). Again, the only difference between *apo* and *holo* forms was the

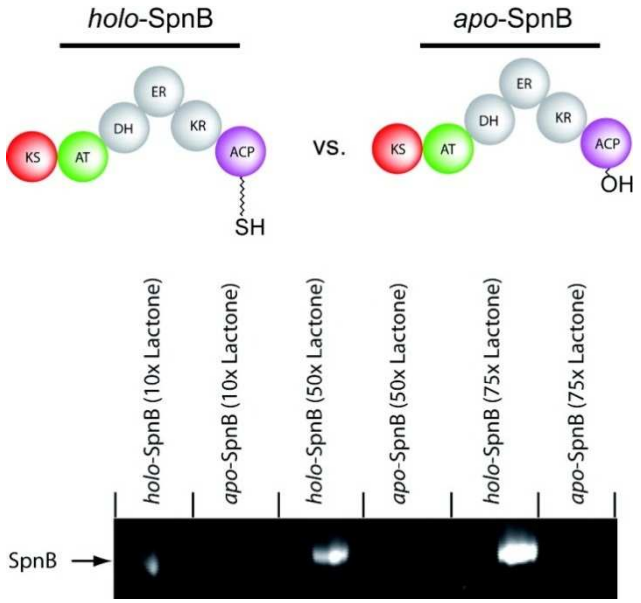

Figure 12. SDS-PAGE analysis of fluorescently labeled SpnB

absence or presence of the Ppant group, respectively. To our satisfaction, only the *holo*-protein showed significant fluorescence indicating that Ppant-thiol is the only competent partner for acylation among a multitude of alternative nucleophiles.Encouraged by these analyses with the alkyne-β-lactone, we began examination of a diverse panel of β-lactone structures. Compounds **2**–**5** were added to *holo*-ACP2 and -3 as well as KS-AT6 at 10- and 50-fold excess and allowed 1h of incubation, followed by proteolysis and LC-MS analyses. Acylation efficiencies, calculated as percent loading, were obtained from peak heights of acylated peptides divided by the total peptide (acylated plus unreacted) peak heights for a given LC-MS run (**Table 1**).

Table 1. Loading of ACP2, ACP3, and KS-AT6 with β-lactone

		loading, %		
compd	**equiv of lactone**	**ACP2**	**ACP3**	**KSAT6**
1	10	33	55	7
	50	93	97	64
2	10	78	64	26
	50	97	97	65
3	10	53	62	41
	50	96	94	62
4	10	8	13	6
	50	32	36	14
5	10	7	15	27
	50	28	35	58

The series of β-lactones tested with the ACP and KS showed different levels of acylation but an interesting trend emerges from this study. The monosubstituted, alkyl-lactones, compounds **1**–**3**, are quite reactive toward both ACPs tested. In contrast, the disubstituted lactone, compound **5**, shows little ACP-acylation activity even at 50 equiv. KS-AT6, on the other hand, is more reactive than ACP2 and -3 toward compound **5** but significantly less reactive with compounds **1**–**3** than the ACPs. Finally, the monosubstitued, aromatic lactone, compound **4**, reacts sluggishly with both ACP and KS. These data offer promising preliminary evidence that β-lactones can provide an efficient and selective means of acylating carrier proteins. High excess

of lactones required for ACP-saturation was a primary concern. We wanted to address this issue to improve the performance but given the limited scope of refining the structure of β-lactones to improve reactivity, we looked towards other suitable electrophilic partners for ACP thiols.

2.2 Experimenatal details

5-hexynal : 20 mL of anhydrous dichloromethane (DCM) were cooled in a dry-ice/acetone bath and oxalyl chloride (13.0 mmol) was added by syringe and stirred for several minutes. DMSO (13.0 mmol) with10% v/v DCM was added dropwise by syringe. After gas evolution ceased, the mixture was stirred for five minutes then 5-hexyn-1-ol (5.20 mmol) was added by syringe and stirred for five minutes.

Triethylamine (26.01 mmol) was added by syringe and the mixture stirred for 15 minutes and warmed to RT. The product was washed with 0.5 M HCl and ½ saturated $NaHCO_3$, and dried over $MgSO_4$. The product was purified by flash chromatography over silica gel using 9:1 Hexanes:EtOAc mobile phase. 5-hexynal: 0.4200 g, 4.37 mmol of product, 84%, colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.811 (s, 1H), 2.619 (t, J = 7.05 Hz, 2H), 2.278 (dt, J = 6.70 Hz, 2.45 Hz,2H), 1.999 (t, J = 2.38 Hz, 1H), 1.858 (quint J = 7.00 Hz, 2H)

^{13}C NMR (100 MHz, CDCl3) δ: 201.719, 83.188, 69.386, 42.530, 20.809, 17.772

***General procedure 1: aluminum-catalyzed generation of β-lactones from aldehydes and acid chlorides*[15].**

A suspension of anhydrous AlCl3 (0.1 eq) and $AgSbF_6$ (0.3 eq) in DCM cooled to -60°C in an ethanol/ethylene glycol/dry ice bath, was added by syringe: N,N'-diisopropylethylamine (DIEA) (1.5 eq), acid chloride (1.5 eq) and aldehyde (1 eq). The mixture was stirred for five hours at -

60°C then filtered through a silica plug. Then product was purified by flash chromatography over silica gel with a hexanes:ethyl acetate mobile phase.

General procedure 2: Nucleophile-catalyzed production of β-lactones from aldehydes and acid Chlorides [16]

For some reactions DABCO was substituted for the quinine catalyst, owing to the similar nature of the nucleophillic substituent.

To a solution of trimethylsilyl quinine ($TMSq_3$) (0.1 eq) or (DABCO) (0.2 eq) and $LiClO_4$ (1.0 eq) in 30 mL of DCM and 10 mL of Et_2O cooled to -78°C in a dry ice-acetone bath, was added via syringe DIEA (3.0 eq), and aldehyde (1 eq). Acid chloride (1 - 2 eq diluted in DCM) was added dropwise over ca. 3 hours. The mixture was stirred for 8 hrs at -78°C then diluted with an equal volume of ether and allowed to come to room temperature. The mixture was filtered through a silica plug then purified by flash chromatography over silica gel with a hexanes:ethyl acetate mobile phase.

4-(pent-4-yn-1-yl)oxetan-2-one (1): General Procedure 1: $AlCl_3$ (0.0427 mmol), $AgSbF_6$ (0.1281 mmol), acetyl chloride (0.5976 mmol), DIEA (0.6656 mmol), 5-hexynal (0.4300 mmol). 3: 0.2389 mmol (56%), pale yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.567 – 4.539 (m, 1H), 3.529 (dd, J = 16.76 Hz, 1.76 Hz, 1H), 3.129 (dd, J = 16.29 Hz, 1.67, Hz, 1H), 2.286 (dt, J = 6.61 Hz, 1.46 Hz, 2H), 2.001 (t, J = 2.4, 1H), 1.720 – 1.644 (m, 4H).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 168.101, 83.236, 70.785, 69.355, 43.029, 33.590, 23.867, 17.982.

Hi-res FAB MS calculated m/z for $C_8H_{11}O_2$ [M+H]+ = 139.075356. Observed m/z = 139.0766.

4-propyloxetan-2-one (3): General Procedure 1: $AlCl_3$ (0.99 mmol), $AgSbF_6$ (2.9 mmol), acetyl chloride (11 mmol), DIEA (11), butanal (9.8 mmol). 2: 8.134 mmol (83%), colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.530 (dt, J = 2.96 Hz, 5.76 Hz, 1H), 3.522 (dd, J = 16.31 Hz, 5.78 Hz,

1H), 3.067 (dd, J = 16.29 Hz, 4.28 Hz), 1.1.871 – 1.838 (m, 1H), 1.833 – 1.745 (m, 1H), 1.56 – 1.36 (m, 1H), 0.986 (t, J = 7.40 Hz)

^{13}C NMR (100 MHz, $CDCl_3$) δ: 166.477, 69.238, 41.036, 34.826, 16.429, 11.802

4-(4-nitrophenyl)oxetan-2-one (4): General Procedure 2: DABCO (0.6 mmol), DIEA (9 mmol), $LiClO_4$ (3 mmol), p-nitrobenzaldehyde (3.0 mmol), acetyl chloride (3.0 mmol). N: 1.35 mmol (45%), pale yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.30 (d, J = 8.88, 2H), 7.60 (d, J = 8.46 Hz, 2H), 5.63 (dd, J = 6.3 Hz, J =4.5 Hz, 1H), 4.04 (dd, J = 16.4, J= 6.3, 1H), 3.44 (dd, J = 16.5, J = 4.5, 1H)

^{13}C NMR (100 MHz, $CDCl_3$) δ: 166.51, 148.42, 144.40, 126.50, 124.36, 69.51, 46.90

3-methyl-4-propyloxetan-2-one (5): General Procedure 2: TMSq (0.3 mmol), DIEA (7.5 mmol), $LiClO_4$ (3.0 mmol), butyraldehyde (3.0 mmol), propionyl chloride (3.0 mmol). N: 1.68 mmol (56%), colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.60 – 4.55 (m, 1H), 3.79 – 3.17 (m, 1H), 1.80 – 1.69 (m, 1H), 1.68 – 1.61 (m, 1H), 1.59 – 1.49 (m, 1H), 1.47 – 1.38 (m, 1H), 1.28 (d, J = 7.8 Hz, 1H), 1.00 (t, J = 7.4 Hz, 1H)

^{13}C NMR (100 MHz, $CDCl_3$) δ: 172.86, 75.56, 47.22, 32.00, 18.79, 13.79, 8.06

Sulforhodamine-B Azide (SrB-A): 3-azido-1-aminopropane[18] (0.22 mmol) and triethylamine (0.5 mmol) was dissolved in 1 mL of 5:1DCM:DMF and cooled to 0°C in an ice-water bath. Sulforhodamine B sulfonyl chloride [mixture of ortho- and para- sulfonyl chloride isomers] (0.20 mmol) was added portion-wise over ca 30 min and stirred overnight at room temperature. The product was purified by flash chromatography over silica gel with 90:5:5 DCM : acetonitrile : methanol mobile phase. The ortho isomer was distinguished by its reversible color change in in pH 9.0 buffer[19]. Para isomer (SrB-pA): 17%, ortho isomer (SrB-oA) 14%. Srb-oA was found to perform best in the click reaction and was utilized for labeling experiments.

NMR spectra are of poor quality due to long relaxation times. Product is pure by LC-MS.

Hi-res FAB MS calculated m/z for $C_{30}H_{37}N_6O_6S_2$ [M+H]+ = 641.221049. Observed m/z = 641.2216.

3. Plasmid Construction

pCAD01 was prepared from an E. coli optimized synthetic construct (DNA2.0, Menlo Park, CA). The synthetic gene was excised from the shipping vector via flanking NdeI and NotI restriction sites and ligated into pET21b.

4. Protein Expression and Purification

SpnB was expressed from pCAD01. KSAT3 and KSAT6 were expressed from pAYC02 and pAYC11[20]respectively. ACP2 was expressed from pNW6[21], ACP3 was expressed from pVYA05[22]. Apo SpnB and ACPs were expressed in E. coli BL-21 cells and Holo SpnB and ACPs were expressed in E.Coli BAP-1[23]. pCAD01, pAYC02, and pAYC11 contain ampicillin resistance vectors, pNW6 and pVYA05 contain kanamycin resistance vectors.

General Procedure for Protein Expression and Isolation

Bacteria were grown in 1 L shake cultures of LB-antibiotic media at 37°C in a New Brunswick Scientific Excella E24 Incubator Shaker until the OD600 was between 0.6 and 0.8. Overexpression was induced with 200 µL of 1 M IPTG and carried out at 18°C for 18 hours, after this point all work was carried out at 4°C. Cells were pelleted by spinning at 3000 RPM for 10 minutes in a Sorvall RC6 Plus with a FiberLite F21S-8x50 rotor and resuspended in 50 mL of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium Na_3VO_4, 1 µg/mL leupeptin, pH 7.5). Cells were lysed using a Misonics ultrasonic converter without microtip, amplitude 30 for five 30 second intervals with a 60 second cool down period between each. Lysed cells were spun at 10,000 rpm and with a FiberLite F10S5 6x500 Y rotor for 60 minutes. The lysate was equilibrated

with 3 mL of PerfectPro Ni-NTA bead slurry for 60 minutes by stirring with a PTFE-coated stir bar at 60 RPM for 60 minutes. The lysate was then poured into a 15 mL column and the supernatant eluted. The column was then washed with two 15 mL portions of wash buffer (50 mM phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0), and eluted with 3 mL of elution buffer (50 mM phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0). The purified protein was loaded into an Amicon Ultra centrifugal concentrator and diluted to 15 mL with storage buffer (100 mM Tris, 1 mM EDTA, 1 mM dithioerythritol, 10% glycerol pH 8) and spun at 3000 rpm in Eppendorf Centrifuge 5810 R with swinging-bucket rotor. Dilution and filtration was repeated a total of three times. Protein concentration determined by Bradford assay, average concentration was approximately 500 μM. Proteins were flash frozen and stored at -80°C until use.

5. Loading Proteins with β-lactones

Labeling reactions for gel assays were performed at 20 μL total volume, reactions for LC-MS were performed at 50 μL total volume. Final concentrations reported in procedure.

Loading ACPs

Phosphate buffer (100 mM, pH 7), tris(2-carboxyethyl)phosphine (2.5 mM), and protein (0.025 mM) were reacted at ambient temperature for 15 min. Then β-Lactone was added to the appropriate concentration (1, 5, 10, 20, 50, or 75x with respect to protein for saturation experiments, 10 or 50x with respect to protein for loading experiments) and the mixture reacted at ambient temperature for 60 min.

Loading KSATs

Phosphate buffer (100 mM, pH 7), tris(2-carboxyethyl)phosphine (2.5 mM), and KSAT (0.025 mM) were reacted at ambient temperature for 15 min, then β-Lactone (2.5 mM) was added (1, 5, 10, 20, 50, or 75x with respect to protein for saturation experiments, 10 or 50x with respect

to protein for loading experiments) and the mixture incubated at ambient temperature for 60 min.

Loading SpnB

Phosphate buffer (100 mM, pH 7), tris(2-carboxyethyl)phosphine (2.5 mM), and SpnB (0.015 mM) were reacted at ambient temperature for 15 min, then β-Lactone was added (10, 50, or 75x with respect to protein) and the mixture incubated at ambient temperature for 60 min.

Acyl Transfer

Phosphate buffer (100 mM, pH 7), tris(2-carboxyethyl)phosphine (2.5 mM), and ACP (0.025 mM) were reacted at ambient temperature for 15 min. Then β-Lactone (10 equivalents) was added and the mixture reacted at ambient temperature for 60 min. Then KSAT was added and the mixture incubated at ambient temperature for 60 min.

6. Chromophore Attachment

The reaction was carried out at 25 µL, final concentrations reported. SrB-oA, (2x alkyne concentration), sodium ascorbate (1 mM), and copper (II) sulfate (1 mM) were added to samples which had been labeled with 1. The reaction was performed at ambient temperature for 60 minutes.

7. Gel Assay

Labeled samples were diluted to 35 µL with gel-loading buffer. ACPs and KSATs were separated by 12.5% SDS-PAGE with 5% stacking gel (100 V, 50 mA, 135 min). SpnB was separated by 4 – 20% gradient HEPES-PAGE (100 V, 50 mA, 90 min). Gels were developed in 10% acetic acid to visualize Srb-oA. Srb-oA labeled proteins were imaged on a BioDoc-It Imaging System with UVtransilluminator. Total protein was stained using GelCode Blue.

8. Proteolysis

Promega Sequencing Grade Modified Trypsin was added to prepared samples so that the final

trypsin:ACP ratio was 1:50 (w/w). The mixture was incubated at 30°C for 18 hours. Digestion was quenched by addition of an equal volume of 10% formic acid. Digests were flash frozen in N_2(l) and stored at -20°C until analysis.

9. LC-MS

Separation was performed with a Waters 1525 system. The gradient employed was A = water + 0.1% formic acid, B = acetonitrile + 0.1% formic acid, 5-95% B over 60 min with a Vydac 218TP C18 5u column (4.6 x 150 mm). Mass spectra were acquired with a Waters Micromass ZQ mass detector in EI+ mode: Capillary voltage = 3.50 kV, cone voltage = 30 V, extractor = 3 V, RF lens = 0.0 V, source temp = 100°C, desolvation temp = 200°C, desolvation gas = 300 L/hr, desolvation gas = 0.0 L/hr The system was operated and spectra were processed using the Waters Empower software suite.

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CHAPTER 3

β-LACTAMS AS VERSATILE REAGENTS FOR ACYL CARRIER PROTEIN LABELING

3.1 Introduction

As introduced in the previous chapter, we began with a simple idea of using electrophilic β-lactones as acylating agents for ACPs.[1] Our hypothesis was that the resulting ACP-bound β-hydroxythioester would represent a common structural motif in modular polyketide biosynthesis . A panel of the substituted lactones was examined for selectivity for the Ppant-thiol over competing nucleophiles, including the KS active site. All of the lactones investigated showed some level of Ppant-acylation ,monosubstituted β-lactones were generally both more reactive and selective. However, it emerged that even the best acylating agents required a very large excess to obtain saturation of the ACPs, indicating that the reactivity of lactones needed improvement. With limited scope of structural modifications given the lactone structure, we were compelled to consider alternate electrophiles for future experimentation. With the purpose of improving upon the reactivity of β-lactones, we explored the idea of using β-lactams as electrophilic parners for ACPs. A series of *N*-activated β-lactams were prepared to examine site-specific acylation of the phosphopantetheine-thiol.

An initial concern was that increased reactivity may result in higher off-target interactions with competing nucleophiles, such as the KS-active site, and decreased selectivity for the targeted Ppant group. To circumvent competition from other nucleophiles for initial experimentation, we adapted the use of separated stand-alone domains of ACP and KS-AT[2-5], as introduced and discussed in Chapter 1. The separated domains retain their function when reintroduced *in vitro*. As a result, the challenge of designing Ppant-acylating agents is reduced to

one of selectivity for *holo*-ACP over *apo*-ACP alone since the KS domain can be introduced after ACP-loading has occurred without limiting the scope of its utility.

In this manner, by using separated domains, we began to explore β-lactams as thiol -reactive acylating agents for isolated ACP domains. Relative to β-lactones, the amide nitrogen of β-lactams offers an additional site for ring substitution that is electronically coupled to the carbonyl, thus providing a reliable means of tuning electrophilicity. The experimental set-up descrbed here is used to establish *N*-activated β-lactams as efficient and versatile acylating agents for ACP domains. Further along, we wished to increase our understanding of KS-ACP recognition and intermodular polyketide transfer between these components, for which, fluorescence and affinity labeling applications were designed.

3.2 Results and discussion

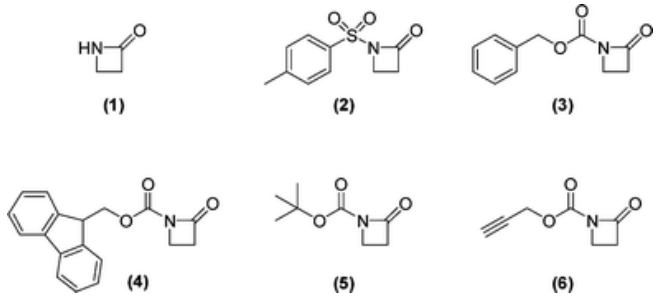

Figure 13. N-activated β-lactams

β-lactams were employed as electrophilic partners as they were expected to have a reactive advantage relative to β-lactones through appropriate activation of the ring nitrogen. [6] To test this hypothesis, a panel of diverse β-lactams were prepared by introducing common nitrogen -protecting groups onto the parent azetidinone (compound **1**) *via* established methods to produce compounds **2–5** (**Figure 13.**)[7-10].To establish the extent of acylation, saturation curves were generated by varying the number of equivalents of each lactam relative to isolated

holo-ACP2 (module 2) and *holo*-ACP3 (module 3) from 6-deoxyerythronolide B synthase (DEBS) in analogous fashion to our previous work with β-lactones.[1] In general, it was performed by incubating the ACP with the lactam for 1 h at room temperature. The mixture was subjected to trypsin digestion and subsequently quenched with formic acid. The resulting array of peptide fragments was analyzed by LC-MS (**Figure 14**). The extent of loading was estimated from the ratio of acylated Ppant-containing peptide to total Ppant-containing peptide , assuming similar ionization potentials. Acylation experiments showed that compounds **2–5** exhibited excellent acylation efficiencies with both *holo*-ACPs(**Table 2,3**). All compounds, with exception of Boc-activated lactam(compound 5), showed around 80% loading at a low 10 equivalents (**Figure 14.**). To demonstrate the benefit of using lactams, a direct competition between compound **6** and the most reactive β-lactone from our previous study, 4-methyloxetan-2-one was performed by treating ACP2 simultaneously with 10 equivalents of each for 1 h. The acylated-ACP was trypsinized and the resulting peptide fragments were analyzed by LC-MS which clearly demonstrates a reactive advantage of β-lactams for acylation of *holo*-ACP (**Figure 15**.). Again, in

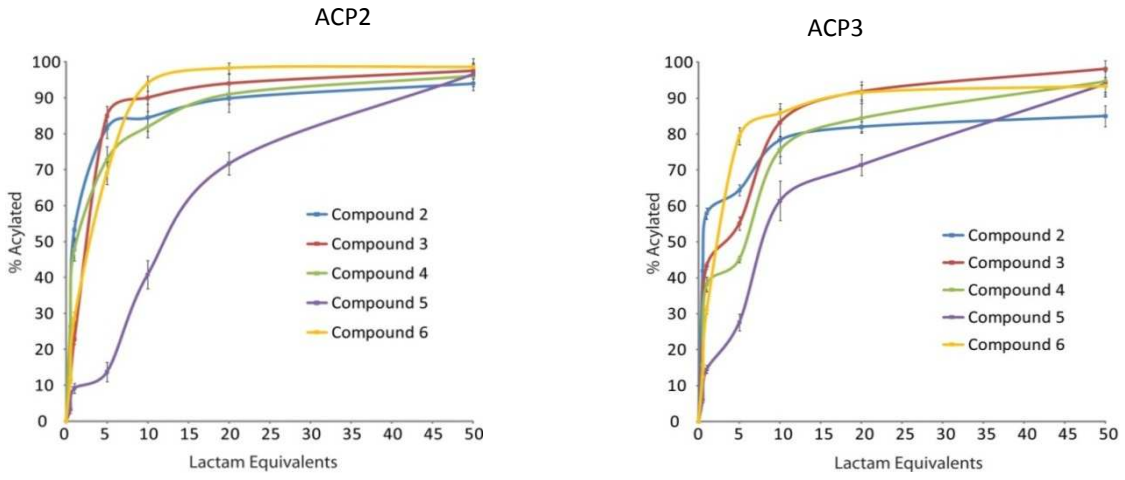

Figure 14. Saturation curves for β-lactam loading

Determined by tandem proteolysis-mass spectrometry for ACP2 (Top) and ACP3 (Bottom) with compounds 2-6. Equivalents of lactam are per protein molecule. Lines are added for clarity. Error bars represent one standard deviation (n=3).

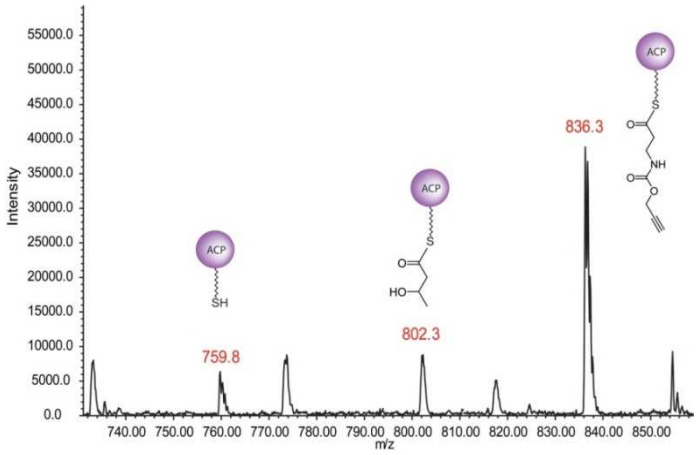

Figure 15. β-lactams vs. β-lactone ACP-acylation

the absence of any solvent-exposed cysteine residues, the Ppant-thiol is the only competent nucleophile present as these ACP.

Table 2. ACP2 loading with β-lactams

Equivalents	Compound 2					Compound 3					Compound 4				
				St. Dev	Avg.				St. Dev	Avg.				St. Dev	Avg.
0.5	12.3	15.0	18.9	2.7	15.3	9.3	12.0	13.9	1.9	11.6	27.0	29.0	23.0	2.5	26.4
1.0	50.2	54.2	56.1	2.4	53.3	21.1	23.7	24.3	1.4	22.8	47.2	51.8	44.0	2.5	47.8
5.0	78.5	79.9	85.6	2.9	81.6	82.3	85.4	89.0	2.7	85.0	73.5	68.0	76.9	3.2	72.8
10.0	80.5	83.4	89.6	3.8	84.5	84.4	92.0	93.0	3.8	90.0	82.0	85.4	78.0	3.0	81.9
20.0	84.5	92.4	93.0	3.9	89.9	90.4	95.4	96.4	2.7	94.0	88.1	90.0	94.7	2.7	91.0
50.0	92.0	93.6	96.5	1.9	94.0	94.5	98.7	99.5	2.2	97.5	92.0	98.4	98.0	2.9	96.1

Equivalents	Compound 5					Compound 6				
				St. Dev	Avg.				St. Dev	Avg.
0.5	2.1	2.9	4.8	1.1	3.3	10.4	12.0	13.4	1.2	11.9
1.0	7.5	8.9	11.1	1.4	9.2	26.0	29.0	30.6	1.9	28.5
5.0	9.8	15.3	15.8	2.7	13.7	65.2	69.0	72.9	3.1	69.0
10.0	36.7	39.8	45.9	3.9	40.8	91.1	94.9	96.0	2.1	94.0
20.0	68.7	70.4	76.3	3.2	71.7	96.2	98.9	99.5	1.5	98.3
50.0	98.4	97.8	93.1	2.8	96.6	96.8	98.9	99.8	2.4	98.6

Table 3. ACP3 loading with β-lactams

Equivalents	Compound 2					Compound 3					Compound 4				
				St. Dev	Avg.				St. Dev	Avg.				St. Dev	Avg.
0.5	41.6	39.8	42.9	1.2	41.9	18.6	21.0	22.5	1.6	20.7	18.0	16.5	13.0	2.1	15.8
1.0	58.0	59.9	56.0	1.6	57.9	42.1	43.7	44.4	1.0	43.3	38.0	41.0	36.0	2.1	38.2
5.0	62.5	64.6	66.2	1.5	64.4	52.5	55.8	56.9	1.9	55.1	46.0	44.0	45.8	0.9	45.2
10.0	73.1	77.4	84.3	4.6	78.3	78.0	85.8	86.2	3.8	83.2	76.0	71.0	80.0	3.7	75.6
20.0	80.2	81.9	83.7	1.4	82.0	89.9	89.7	95.7	2.8	91.8	85.0	79.0	89.0	4.1	84.4
50.0	81.4	85.8	88.3	2.9	85.0	94.8	98.9	99.4	2.4	98.1	27.0	29.0	23.0	3.7	94.7

Equivalents	Compound 5					Compound 6				
				St. Dev	Avg.				St. Dev	Avg.
0.5	5.2	6.0	7.2	0.8	6.0	11.4	12.1	14.2	1.2	12.5
1.0	14.0	13.4	16.0	1.1	14.5	28.6	32.0	33.1	1.9	31.1
5.0	24.5	28.0	30.2	2.3	27.6	75.6	81.2	83.1	3.1	79.4
10.0	55.0	61.0	68.4	5.5	61.4	83.1	86.9	87.9	2.1	85.8
20.0	67.5	72.9	74.1	2.9	71.4	89.4	92.0	93.2	1.5	91.6
50.0	89.4	94.3	98.0	3.5	93.9	90	94	95.6	2.4	93.4

A gel-based fluorescent assay was used, based on work by Sieber and coworkers, to further amine selectivity for *holo*- over *apo*-ACP[11]. A [3 + 2] cycloaddition (click reaction) was used for coupling acylated ACPs to a rhodamine-based fluorophore.[12–14] ACP2 and ACP3 (both *holo* and *apo*) were mixed with 1, 5, and 10 equivalents of **6** and reaction continued at room temperature for 1 h. The click step was performed with addition of Rhodamine azide along with all necessary click reaction components and the resulting protein products were analyzed *via* SDS-PAGE . Bright bands were observed for the *holo*-ACPs while *apo*-ACPs provided only background fluorescence (**Figure 16**.). Remarkably, fluorescence can be easily observed at an impressive 5 equivalents of lactam for both ACPs, indicating the extent of reactivity.

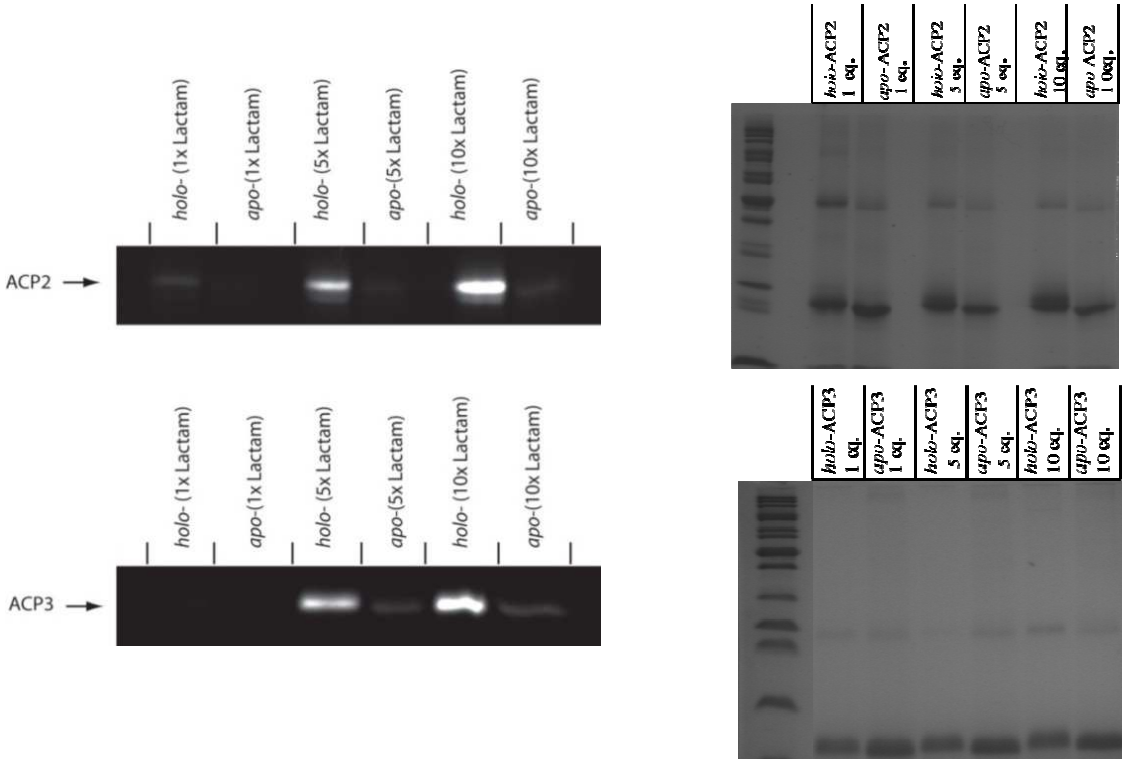

Figure 16. Fluorescence SDS-PAGE analysis of DEBS ACP2 and ACP3

In order to explore Ppant selectivity and further demonstrate utility of the alkyne handle, we turned our focus to mixtures of *apo* and *holo* ACP2. We wanted to test whether *holo*-ACP2 could be isolated from a mixture of *apo* and *holo* by an affinity label conjugated to compound **6**. A biotin -streptavidin interaction was used as on bio-orthagonal agent for ACP purification as this system (1) has been used extensively for protein separations[15–18] and (2) relies on a small molecule that we envisioned could readily be conjugated to the lactam warhead. As proof of principle and to assess the efficiency of each step, we began with samples of pure *holo*-ACP. Initially, attempts to pre-conjugate compound **6** with the biotin azide followed by 1 h reaction with the ACP resulted in low yields of the acylated protein . Another observation was competition from other proteinaceous nucleophiles for longer reaction times which forced us to consider an alternative order of addition.

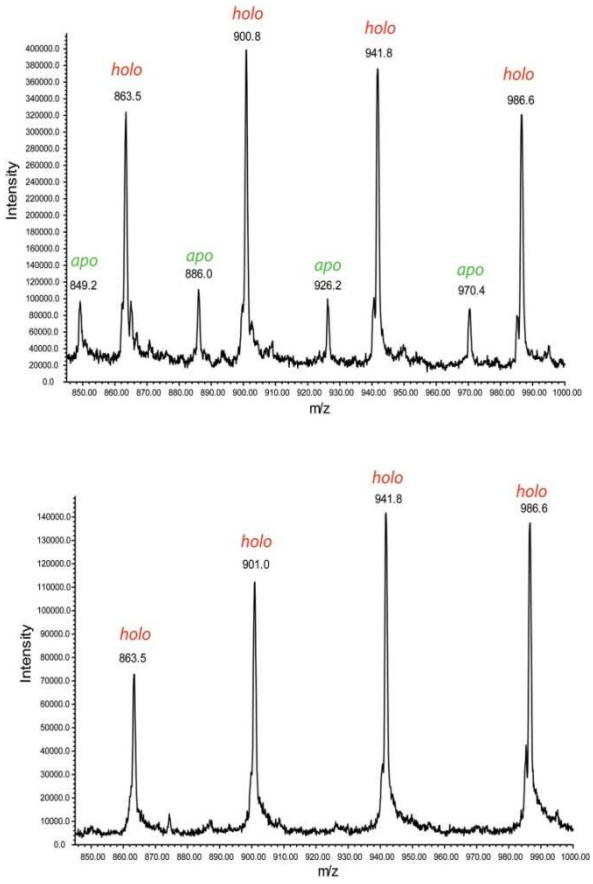

Figure 17. Whole protein mass spectra of ACP2 purification strategy

(top) an initial 80:20 (*holo*:*apo*) mixture of ACP2 and (bottom) the result of β-lactam based purification of *holo*-ACP2. The lack of *apo*-ACP2 in the latter indicates that the β-lactam (compound **6**) is highly selective for *holo*-ACP2

The [3 + 2] cycloaddition of the biotin azide with pre-acylated *holo*-ACP resulted in 30% recovery following streptavidin–sepharose binding and subsequent treatment of the bound material with hydrazine, which restores the Ppant-thiol *via* nucleophilic attack on the thioester carbonyl. However, the samples has to be subjected to filtration twice prior to treatment with the streptavidin beads, to remove excess lactam and biotin following the initial acylation and subsequent [3 + 2] cycloaddition. The protein concentration was analyzed at each stage which revealed that the primary source of ACP loss in the process was these filtration steps. In fact, more than 60% of the protein subjected to the streptavidin was recovered upon treatment with

hydrazine while almost half of the initial mixture is lost to the filtration membrane before this step. It is expected that upon scale-up, where small losses of protein to filtration are less significant, percent recovery will be drastically improved. As proof of principle, however, we were confident that these recovery levels would suffice for further experimentation.

The *holo* (Ppantylated)-form of ACP is resulted due to overexpression of ACP in BAP1, an engineered strain of *E. coli* with a phosphopantetheinyl transferase embedded in the genome. However, as expression levels increase, the transferase performance suffers resulting in mixtures of *holo*- and *apo*-ACP. We examined the viability of our approach for separating *holo*- from *apo*-ACP with a 80 : 20 (*holo* : *apo*) mixture of ACP2, a typical ratio observed from overexpression of ACP in BAP1, and subjected it to the same process described above. Analysis of the final solution obtained from hydrazine treatment of the bound material by LC-MS revealed only *holo*-ACP2 (**Figure 17**.). Taken together with the fluorescence assay, these experiments nicely illustrate the remarkable Ppant-selectivity and versatility of compound 6.

We wanted to confirm that the purification process did not alter the protein structure. It was confirmed by examining the ability of eluted *holo*-ACP2 to accept a methylmalonyl-CoA unit from an acyltransferase. Methylmalonyl-CoA and purified *holo-ACP* was added to a buffered solution of DEBS module 6 ketosynthase-acyltransferase didomain[5,19-21] ([KS6AT6]). After 30 min of reaction time, the mixture was subjected to trypsinolysis and the resulting peptide fragments were separated and analyzed *via* LC-MS (**Figure 18.**). The prominent peak at 809.1 mass units suggests that the purified ACP remains competent for AT-ACP methylmalonate transfer as seen previously.[21]

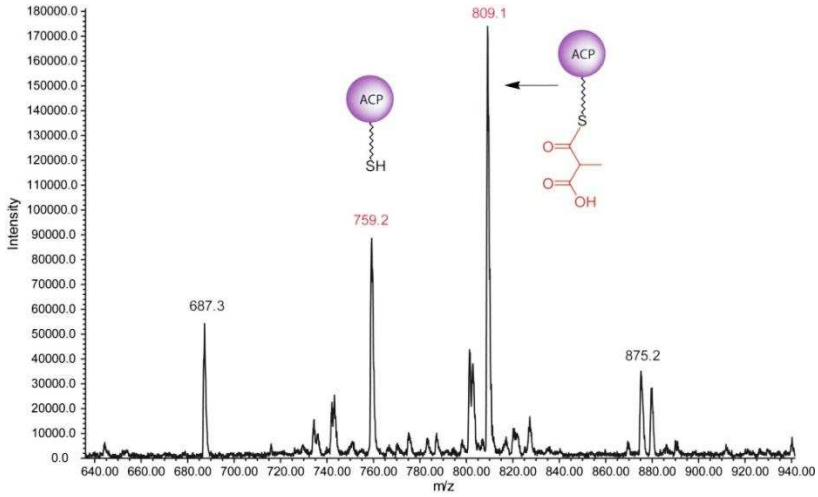

Figure 18. Mass spectrum of methylmalonyl-CoA transfer to purified ACP

In this manner, it was established that carrier proteins could be efficiently acylated with β-lactams. However, to demonstrate the utility of these reagents for PKS examination, a direct comparison to traditional small molecule probes was necessary. In particular, we were interested in ACP to KS transfer of the β-aminothioester product (**Figure 19.**). Polyketide intermediate transfer between ACP and KS plays a critical role in product generation for all PKS systems and acts as a primary determinant for proper flow of intermediates during polyketide biosynthesis. As discussed in Chapter1, *N*-acetylcysteamine thioester (SNAc) mimics the terminal portion of the Ppant group and is most commonly used for direct acylation of KS active sites.[22–25] A gel-based fluorescence assay was employed to qualitatively determine the kinetic competency of our lactam -derived acyl-ACPs, relative to propionyl-SNAc and the potent KS-inhibitor, cerulenin[26].

Acylation of *holo*-ACP2 was executed as before using 10 equivalents of compound **6** followed by Cu-mediated [3 + 2] cycloaddition with rhodamine azide. The resulting acyl-ACP was incubated with [KS6AT6] for 1 h at room temperature with or without competing KS-acylating/alkylating agents to explore ACP to KS transfer (**Figure 20**.). From these data , it is clear

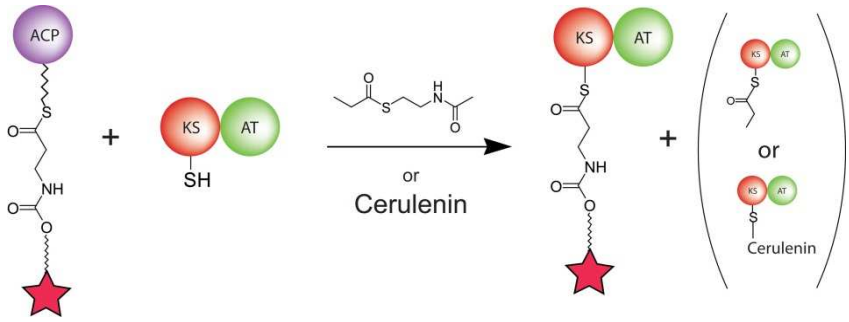

Figure 19. Schematic for fluoroscence assay for ACP to KS acyl transfer.

that fluorescence transfers from the ACP to KS in the absence and presence of both cerulenin and propionyl-SNAc, indicating that the lactam -derived acyl-ACPs are, at a minimum, competitive with traditional small molecule probes (Lanes 2, 4, and 6). The lack of KS-fluorescence observed when [KS6AT6] is pre-treated with each competitor (Lanes 3 and 5) confirms that transfer is occurring between the Ppant arm and KS active site. Finally, the absence of a fluorescent band when [KS6AT6] is treated directly with the compound **6**-rhodamine click product implies that the ACP is required for transfer to occur (Lane 7). In all, these results suggest that β-lactam-derived, acyl-ACPs behave in analogous fashion to natural polyketide intermediates and are kinetically competitive with traditional PKS probes and inhibitors . Further experimentation to quantitatively determine the transfer rate is currently ongoing in the lab but, at this point, it is clear that activated β-lactams offer a greatly simplified and readily adaptable set of carrier protein modifiers.

3.3 Conclusion

In summary, we have demonstrated the utility of *N*-activated β-lactams for acylation of *holo*-ACPs. The structures examined are significantly more reactive than the β-lactones that we have previously reported and all are available in a single synthetic step from commercially available starting materials. Both carbamate and sulfamate moieties effectively activate the lactam ring for ACP-acylation. The resulting acylation products maintain the thioester linkage common to all polyketide biosynthetic intermediates and the reaction can be readily reversed by treatment with hydrazine.

The applications of β-lactams demonstrated so far just begin to scratch the surface of their capabilities. The *N*-propargyloxycarbonyl group (compound **6**) provides a versatile chemical handle for nearly limitless elaboration of structure. Although we have focused exclusively on proteins involved in polyketide biosynthesis , we expect the general thiol -reactivity of these simple β-lactams to translate well to other systems bearing natural or engineered thiols.

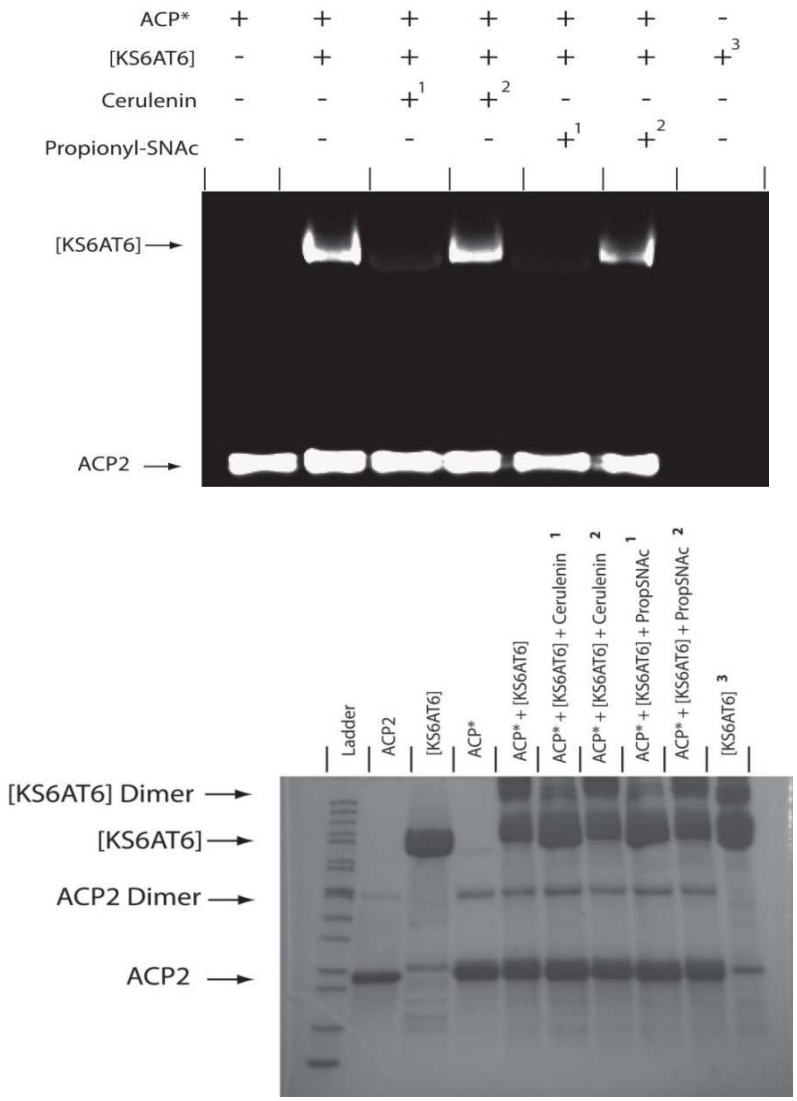

Figure 20. Fluorescent competition assay for ACP to KS acyl transfer.

(Top) Fluorescent ACP2 (ACP*) is prepared via click coupling of rhodamine azide with ACP2, preacylated with compound **6**. ACP2* is then incubated with [KS6AT6] under various conditions. Lanes are marked with the corresponding components for each reaction. [1]{KS6AT6] was mixed with competitor (cerulenin or propionyl-SNAc) for 1 hour prior to addition of ACP* (Lanes 3 and 5). [2]ACP* and competitor were introduced to [KS6AT6] simultaneously (Lanes 4 and 6). [3]{KS6AT6] was mixed with 10 eq of the click product in the absence of ACP2 (Lane 7). SNAc = *N*-acetylcysteimine. (Bottom) PAGE full gel stain.

3.4 Experimental Details

Synthesis

1-[(4-Methylphenyl)sulfonyl]azetidin-2-one[27] (2). 2-Azetidinone (72 mg, 1 mmol) was dissolved in THF (5 mL) at −78 °C. NaHDMS (367 mg, 2 mmol) dissolved in THF (1 mL) was added in portions. 4-Toluenesulfonyl chloride (763 mg, 4 mmol) dissolved in THF (5 mL) was added to the reaction mixture in portions slowly over 15 min. The reaction mixture was stirred until all the starting material was consumed (monitored by TLC), approximately 8 h. The reaction was washed with sodium bicarbonate (10 ml ×3) and extracted with DCM (25 mL ×3). The combined organic layers were dried over Na_2SO_4, and the solvent was removed by rotary evaporation . The crude material was purified by flash chromatography with 1 : 1 hexane : ethyl acetate. Yield: (178 mg, 0.79 mmol) 79% of final product as colorless solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.9–7.87 (d, J = 8.29, 2H), 7.32–7.41 (d, J = 8.48, 2H), 3.67–3.64 (t, J = 5.2, 2H), 3.06–3.02 (t, J = 5.1, 2H), 2.46 (s, 3H).^{13}C NMR (400 MHz, $CDCl_3$) δ: 161.9, 143.4, 128.3, 125.7, 38.0, 34.9, 19.9. IR : v 2973, 1776, 1362, 1154, 682, 98 cm^{-1}, MS $[M + H]^+$ = 227

Benzyl 2-oxoazetidine-1-carboxylate[8] (3). HDMS (250 mg, 1.5 mmol) was dissolved in THF (8 mL) and cooled to −78 °C. *n*BuLi (96 mg, 1.5 mmol) was added dropwise and stirred for 30 min. In a separate RBF, 2-azetidinone (106.6 mg, 1.5 mmol) was dissolved in 5 mL of THF at −78 °C. The contents of the first flask were transferred *via* cannula to the second flask and stirred for 1 h. Benzylchloroformate (256 mg, 1.5 mmol) was added in portions and stirred for 2 h at −78 °C. The reaction mixture was allowed to warm to ambient temperature and stirred for an additional 4 h. The reaction mixture was diluted with water and extracted with DCM (50 mL × 3). Combined organic phases were washed with brine and dried over Na_2SO_4. Solvent was removed

by rotary evaporation . Purification of crude product by flash chromatography with 5 : 1 hexane : ethyl acetate yields (285 mg, 1.39 mmol) 93% of product as an oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.28–7.16 (m, 5H), 5.1 (s, 2H), 3.44–3.4 (t, J = 5.3, 2H), 2.85–2.81(t, J = 5.3, 2H).^{13}C NMR (400 MHz, $CDCl_3$) δ 23.7, 34.7, 36.1, 65.9, 126.1, 126.8, 147.1, 155.0, 167.8. IR : v 3040, 2985, 1811, 1724, 1388, 1330, 1214, 1177, 1120, 1044, 763, 699 cm^{-1}, MS $[M + H]^+$ = 206

(9*H*-Fluoren-9-yl)methyl 2-oxoazetidin-1-carboxylate (4). 2-Azetidinone (72 mg, 1 mmol) was dissolved in THF (5 mL), and LiHDMS (167 mg, 1 mmol) dissolved in THF (2 mL) was added dropwise at −78 °C over a 15 min period and stirred for an additional 30 min. 9-Fluorenylmethyl chloroformate (259 mg, 1 mmol) dissolved in THF (2 mL) was added dropwise at −78 °C. The resulting mixture was stirred for 1 h at −78 °C, then allowed to warm to R.T, and stirred for 1 h further at which point the starting material had been completely consumed (monitored by TLC). The reaction mixture was poured into water and extracted with DCM (3 × 25 mL). The combined extracts were washed with brine and dried over Na_2SO_4. After removal of the solvent by rotary evaporation , the crude reaction mixture was purified by flash chromatography with DCM : ethyl acetate, 95 : 5, producing the product as an off-white solid (62 mg, 0.62 mmol) in 86% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.13 (t, 2H), 3.68 (t, 2H), 4.32 (t, 1H), 4.47 (d, 2H), 7.26–7.80 (m, 8H). ^{13}C NMR (400 MHz, $CDCl_3$) δ 162.1, 148.3, 141.3, 139.4, 126.4, 125.5, 123.5, 118.1, 66.8, 44.3, 34.8, 36.2. IR : v 3015, 2922,1770, 1722, 1449, 1388, 1313,1119, 1044, 964, 738 $cm^{-1.}$ HRMS (EI+) Calcd for $C_{18}H_{15}NO_3$, 293.1052; Found, 293.1046

***tert*-Butyl 2-oxoazetidine-1-carboxylate[28] (5).** 2-Azetidinone (72 mg, 1 mmol) and DMAP (12.2 mg, 0.1 mmol) were dissolved in acetonitrile (5 mL) at 0 °C. Di-*tert*-butyl dicarbonate (218 mg, 1.1 mmol) was added to the reaction mixture in portions. The reaction mixture was stirred at 0 °C for 2 h, allowed to warm to ambient temperature and stirred overnight. The reaction mixture

was diluted with ethyl acetate and washed with 1 N aq. HCl and brine. The aqueous layer was extracted with ethyl acetate (25 mL ×3). Combined organic layers were dried over Na_2SO_4 and the solvent was removed by rotary evaporation . The crude product was purified by flash chromatography using 2 : 1, hexanes : ethyl acetate (154 mg, 0.9 mmol) 90% of product as an oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.56–3.52 (t, J = 5.1, 2H), 3.03–2.87 (t, J = 5.2, 2H), 1.5 (s, 9H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 205.4, 162.1, 146.5, 81.2, 36.8, 35.0, 29.8, 26.2. IR : v 2984, 1795, 1719, 1331, 1157, 1043 cm^{-1}, MS $[M + H]^+$ = 172

Prop-2-yn-1-yl 2-oxoazetidine-1-carboxylate (6). 2-Azetidinone (107 mg, 1.5 mmol) was dissolved in THF (7 mL) at −78 °C. LiHDMS (330 mg, 2 mmol) dissolved in THF (2 mL) was added in portions over 10 min and the reaction mixture was stirred for 30 min. Propargyl chloroformate (181 mg, 1.5 mmol) dissolved in THF (2 mL) was added in portions over 10 min and the reaction mixture was stirred at −78 °C for 2 h then allowed to warm to ambient temperature. The reaction mixture was diluted with water and extracted with DCM (50 mL ×3). The combined organic layers were washed with brine and dried over Na_2SO_4. Solvent was removed by rotary evaporation . Purification of crude product by flash chromatography with 95 : 5 DCM : methanol yields (106 mg, 0.69 mmol) 46% of product as a pale yellow solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.82–4.81(d, J = 23.3, 1H), 3.68–3.65 (t, J = 5.3, 2H), 3.10–3.07 (t, J = 5.3, 2H), 2.55–2.53 (t, J = 2.4, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ 34.9, 36.1, 51.8, 74.0, 146.3, 162.2. IR : v 3251, 2925, 2134, 1782, 1722, 1313, 1187, 1114, 1045, 615 $cm^{-1.}$ HRMS (EI+) Calcd for $C_7H_7NO_3$, 153.0426; Found, 153.0402

Sulforhodamine-B azide (S1)[1]. 3-Azido-1-aminopropane[29] (22 mg, 0.22 mmol) and triethylamine (51 mg, 0.5 mmol) were dissolved in 1 mL of 5 : 1 DCM : DMF and cooled to 0 °C. Sulforhodamine B, sulfonyl chloride [mixture of *ortho*- and *para*- sulfonyl chloride isomers] (115

mg, 0.20 mmol) was added portion-wise over *ca.* 30 min and stirred overnight at room temperature. Solvent was removed *in vacuo*, and the product was purified by flash chromatography over silica gel with 90 : 5 : 5 DCM : acetonitrile : methanol mobile phase . The *ortho* isomer was distinguished by its reversible color change in pH 9.0 buffer .[30] *para* Isomer: (22 mg 0.034 mmol) 17%, *ortho* isomer (18 mg, 0.028 mmol) 14%. Both obtained as a red solid with a metallic green luster. The *ortho* isomer was found to perform best in the click reaction and was utilized for labeling experiments.

NMR spectra are of poor quality due to long relaxation times. Product is pure by LC-MS . IR : *v* 2975, 2100, 2590, 1339, 1180 cm^{-1} HRMS (EI+) Calcd for $C_{30}H_{37}N_6O_6S_2$, 641.2210. Found, 641.2216.

Biotin azide (S2). D(+)-biotin (73 mg, 0.300 mmol) was dissolved in 3 mL of DMF by briefly heating the mixture with a heat gun. The mixture was cooled to 0 °C in an ice-water bath and 6-chloro-1-hexanol (120 mg, 0.900 mmol), and *N*,*N*-dimethylamino pyridine (73 mg, 0.600 mmol) were added. *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (115 mg, 0.600 mmol) was added in portions over *ca.* 60 min and the reaction mixture was allowed to come to room temperature. Stirring was continued for 24 h. When biotin had been consumed (monitored by TLC), the reaction mixture was diluted with ethyl acetate (100 mL). The organic phase was washed with concentrated $NaHCO_3$ (3 × 50 mL), and pH 2 saturated Na_2SO_4 (5 × 50 mL portions). Solvent was removed by rotary evaporation and the material was used without further purification . The material from the ester coupling was dissolved in 5 mL of DMF and NaN_3 (40 mg, 0.600 mmol) and a catalytic amount of KI were added. A reflux condenser was attached and the reaction mixture was heated to 80 °C for 16 h. The material was diluted with 100 mL of ethyl acetate and washed with brine. The solvent was removed by rotary evaporation

and the crude material purified by flash chromatography (5% MeOH in DCM) to yield 67 mg, 0.182 mmol of product (61%) as a waxy pale yellow solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.32–1.52 (m, 6 H) 1.56–1.80 (m, 8 H) 2.33 (t, *J* = 7.45, 2 H) 2.74 (d, *J* = 12.76, 1 H) 2.91 (dd, *J* = 12.82, 4.99, 1 H) 3.16 (ddd, *J* = 8.34, 6.38, 4.74, 1 H) 3.28 (t, *J* = 6.88, 2 H) 4.06 (t, *J* = 6.63, 2 H) 4.31 (ddd, *J* = 7.67, 4.71, 1.14, 1 H) 4.51 (dd, *J* = 7.71, 5.05, 1 H) 5.67 (s, 1 H) 6.03 (s, 1 H). ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm) 173.7, 163.7, 65.6, 61.2, 60.8, 56.1, 51.3, 40.5, 33.9, 29.9, 28.7, 28.4, 27.2, 26.3, 25.5, 24.8. IR : *v* 3230, 2934, 2101, 1733,1700, 1264, 1175 cm^{-1}HRMS (EI+) Calcd for $C_{16}H_{27}N_5O_3S$, 369.1848; Found, 369.1814

Propionyl-*N*-acetylcysteamine (Propionyl-SNAc) To a solution of triethylamine (2.80 mmol) in dichlromethane (5 mL) was added propionic acid (1.40 mmol), (3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.40 mmol), 1-hydroxybenzotriazole (HOBt) (1.40 mmol) and N-acetyl cysteamine (SNAc)(1.35 mmol). The reaction mixture was stirred overnight. The organic layer was washed with saturated NaHCO3 solution, 0.1 N HCl solution and brine. It was then dried over anhydrous sodium sulfate, concentrated under vacuum, and purified by flash column chromatography (1 : 1 hexane : ethyl acetate) to provide compound (140 mg, 74%) as a pale yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 0.86(t,*J* = 7.58 Hz, 3H) 1.69 (s, 3H) 2.28 (q, *J* = 7.4 Hz, 2H) 2.72(t, *J* = 6.8 Hz, 2H) 3.07 (q, *J* = 6.6 Hz, 2H) 7.39 (br s, 1H).^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm) 199.4, 170.6, 39.0, 36.8, 28.0, 22.6, 9.4. IR : *v* 3282,2979,1690,1650,1546,1373,1288,1090,935 cm^{-1}MS $[M + H]^+$ = 176

Protein expression and purification

ACP2 was expressed from pNW06,[3]1 ACP3 was expressed from pVYA05.[5] [KS6AT6] was expressed from pAYC11.[20]*apo*-ACPs were harvested from *E. coli* BL-21 and *holo*-ACPs were harvested from *E. coli*. BAP-1[32] pNW6 and pVYA05 contain kanamycin resistant vectors.

General procedure for protein expression and isolation

Bacteria were grown in 1 L shake cultures of LB-antibiotic media at 37 °C in an incubating shaker until the OD600 was between 0.6 and 0.8. Over expression was induced with 200 µL of 1 M IPTG (per liter of culture) and carried out at 18 °C for 18 h. After this point all work was carried out at 4 °C. Cells were pelleted by spinning at 3000 RPM for 10 min and resuspended in 50 mL of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium Na_3VO_4, 1 µg mL^{-1} leupeptin, pH 7.5). Cells were lysed using an ultrasonic converter. The lysate was pelleted at 10,000 rpm for 60 min. The supernatant was equilibrated with 3 mL of Ni-NTA slurry (per liter of culture) for 60 min by stirring with a PTFE-coated stir bar at minimal speed. The mixture was then poured into a fritted column and the supernatant eluted. The resin bed was washed with two 15 mL portions of wash buffer (50 mM phosphate, 300 mM NaCl, 20 mM imidazole , pH 8.0), and eluted with 3 mL (per liter of culture) of elution buffer (50 mM phosphate, 300 mM NaCl, 250 mM imidazole , pH 8.0). The purified protein was loaded into a 3 kDa NMW centrifugal concentrator and diluted to 15 mL with storage buffer (100 mM Tris, 1 mM EDTA, 1 mM dithioerythritol, 10% glycerol pH 8) and spun until the final volume was ≤500 µL. Dilution and filtration was repeated a total of three times. Protein concentration was determined by Bradford assay with a BSA standard curve, average final concentration was approximately 3 mM. Proteins were flash frozen and stored at −80 °C until use.

Proteins acylation with β-lactams

Labeling reactions for gel assays were performed at 20 µL total volume, reactions for LC-MS were performed at 50 µL total volume. Final concentrations reported in procedure. Phosphate buffer (100 mM, pH 7), tris(2-carboxyethyl)phosphine (TCEP) (2.5 mM), and protein (0.025 mM)

were reacted at ambient temperature for 15 min. β-Lactam was added to achieve the appropriate final concentration (0.5, 1, 5, 10, 20, 50x) with respect to protein . The mixture was equilibrated at ambient temperature for 60 min.

β-Lactam/β-lactone competition loading of ACP

10 equivalents of compound **6** and 4-methyloxetan-2-one were mixed and introduced to buffered *holo*-ACP2 for 1 h. The sample was trypsinized analyzed by LC-MS as described previously.[1]

Chromophore attachment

The reaction was carried out at 25 µL, final concentrations reported. **S1**, (2× with respect to **6**), sodium ascorbate (1 mM), and copper(II) sulfate (1 mM) were added to samples which had been labeled with **6**. The reaction was performed at ambient temperature for 60 min.

Gel assay

Labeled samples were diluted to 35 µL with gel-loading buffer . ACP 2 was separated by 12% and ACP 3 with 15% SDS-PAGE , each with a 5% stacking gel run at 100 V, 50 mA, 135 min. Gels were developed in 10% acetic acid to visualize **S1**. Labeled proteins were imaged on a UV-transilluminator. Total protein was stained using Coomassie stain. For the ACP to KS transfer experiments (**Figure.** 8), proteins were separated using a 4–20% gradient HEPES-PAGE gel (100V, 50mA, 90 min).

Proteolysis

Sequencing grade modified trypsin was added to prepared samples so that the final trypsin : ACP ratio was 1 : 50 (w/w). The mixture was incubated at 30 °C for 18 h. Digestion was quenched by addition of an equal volume of 10% formic acid. Digests were flash frozen in liquid nitrogen and stored at −20 °C until analysis.

Affinity purification of *holo*-ACPs

Lactam modification of ACPs

Unless otherwise stated, phosphate buffer refers to 100 mM, pH 7.0 phosphate. 1 mL of an 80 : 20 mixture of *holo* : *apo*-ACP2 (25 µM total protein) was equilibrated at ambient temperature for 15 min in phosphate buffer containing 2.5 mM TCEP. **6** (25× with respect to total ACP concentration) was added and the mixture was equilibrated at ambient temperature for 1 h. To remove excess **6**, the mixture was loaded into a 3kDa NMW concentrator and the volume reduced to 100 µL by spinning in a centrifuge cooled to 4 °C. The mixture was diluted to 500 µL with phosphate buffer , and then concentrated to 100 µL again. This process was repeated a total of 3 times. Protein was removed from the concentrator by inverting it and spinning . The concentrator was then washed several times with phosphate buffer which was added to the protein sample. The filtered protein was reconstituted to 900 µL.[*3 + 2*] *Cycloaddition* In a separate microfuge tube, DMSO (100 µL), **S2** (50× with respect to protein), THPTA[33](1.1 mM), NaAsc (10 mM), and $CuSO_4$ (1 mM) were combined. Best results are obtained if these reagents are first combined in a separate container and then added to the protein . The combined click reagents are added to the protein from the previous step and allowed to react at ambient temperature for 6 h. Excess biotin reagent was removed using the same procedure for the removal of excess lactam . The modified protein was reconstituted to 1000 µL in phosphate buffer .

Immobilization of modified ACPs

Streptavidin Sepharose High Performance (10× with respect to protein) was washed 3× with 1000 µL of phosphate buffer and the supernatant was removed. The modified protein from the previous step was added to the beads, and the mixture equilibrated at ambient temperature for 60 min with agitation on an orbit shaker. The supernatant was removed and the beads were

washed 5× with 1000 µL of phosphate buffer , 5× with 1000 µL of TRIS buffer (100 mM, pH 9.0), and once with 1000 µL of 100 mM TRIS, +10 mM N_2H_4, pH 9.0.

Elution of immobilized ACPs

The washed beads from the previous step were resuspended in 1000 µL of 100 mM ammonium formate containing 100 mM N_2H_4 (100 mM pH 9.0) and equilibrated overnight at 4 °C with agitation on an orbit shaker. Eluted protein was concentrated in a 3kDa NMW concentrator and subjected to buffer exchange for further experiments, or combined with TCEP for direct infusion LC-MS experiments.

Fluorescent assay for ACP to KS transfer

Holo-ACP2 (25 µM) was labeled with 10 equivalents of **6** followed by [3 + 2] cycloaddition with **S2** as described above. For transfer of the fluorescent product, [KS6AT6] (25 µM) was introduced to acyl-ACP2 (25 µM) and incubated for 1 h. For lanes 3 and 5 (**Figure.** 9), [KS6AT6] was pretreated with cerulenin (5 mM) or propionyl SNAc (10 mM) for 1 h before introducing it to acyl-ACP2. For lanes 4 and 6, acyl-ACP2 (25 µM) was mixed with cerulenin (5 mM) or propionyl-SNAc (10 mM) followed by 1 h incubation with [KS6AT6] (25 µM). The [KS6AT6] control (lane 7) was executed under the same acylation and click conditions as in other samples but without the ACP or small molecule competitors.

LC-MS

Separation was performed with a Waters 1525 system. The gradient employed was A = water + 0.1% formic acid, B = acetonitrile + 0.1% formic acid, 5–95% B over 60 min with a Waters XBridge C18 5u column (4.6 × 150 mm). Mass spectra were acquired with a Waters Micromass ZQ mass detector in EI+ mode: Capillary voltage = 3.50 kV, cone voltage = 30 V, extractor = 3 V, RF lens = 0.0 V, source *T* = 100 °C, desolvation *T* = 200 °C, desolvation gas = 300 L h^{-1},

desolvation gas = 0.0 L h^{-1} The system was operated by and spectra were processed using the Waters Empower software suite.

3.5. References

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CHAPTER 4

A MECHANISM-BASED FLUORESCENT TRANSFER ASSAY FOR EXAMINING KETOSYNTHASE SELECTIVITY

4.1. Introduction

As discussed in detail in earlier chapters, the field of polyketide engineering has faced a constant struggle to overcome often strict substrate selectivities of the critical biosynthetic enzymes.[1–7] In case of modular PKS systems, the polyketide product flow along the assembly line is governed by shuttling of intermediates between the acyl carrier protein (ACP) and

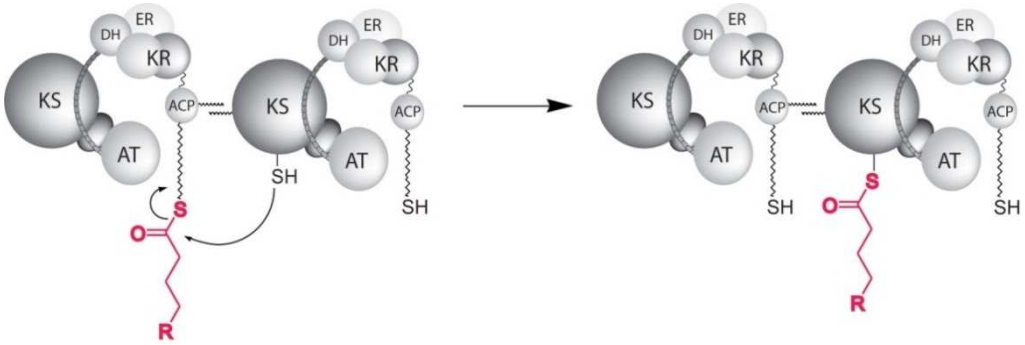

Figure 21. Intermodular transfer in modular polyketide synthases.
A polyketide intermediate is passed from an upstream ACP to a downstream KS via the phosphopantetheine arm of *holo*-ACP. Often the substrate selectivity of the acceptor KS domain determines the efficiency of this process and controls the flow of intermediates down the assembly line. KS = ketosynthase, ACP = acyl carrier protein, AT = acyltransferase, DH = dehydratase, ER = enoyl reductase, KR = ketoreductase.

ketosynthase (KS) domains (**Figure 21**.).[8-11] The KS occupies a central role in this crucial process and has been the focus of numerous studies with the hopes of both establishing a better understanding of natural systems and improving our ability to manipulate them.[12–15] In its simplest form, this requires a means of accurately differentiating between substrates that acylate the KS active site-cysteine and those that do not.

As described previously, radiolabelling traditionally used to examine KS active site loading has significant drawbacks, including limited scope, reproducibility, and cost that can severely constrain the scope of such analyses. More recently, a number of groups have developed mass spectrometry-based techniques for examining KS active site acylation.[13,14,16] One particularly popular approach utilizes tandem proteolysis/LC-MS to tease out differences in loading propensities for various substrates. Despite the many advantages compared to radiolabeling, MS-based methods still suffer from a number of important shortcomings. It is abundantly clear that techniques capable of providing easy and reliable access to KS selectivity profiles are essential for continued progress in this field. With hosts of new biosynthetic systems being uncovered with constant advances in genomic mining, the need for simple, cost-effective means of characterizing them has never been greater. We were interested to develop an alternative means of directly determining KS substrate selectivity using readily accessible, inexpensive reagents and equipment. Such a method would greatly facilitate the process of high-throughput PKS investigation and allow for broadened exploration of the rules of substrate recognition that govern small molecule output.

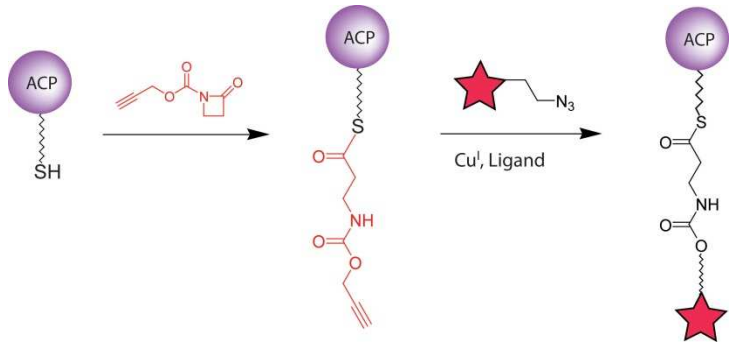

Figure 22. Site-selective ACP-acylation using *N*-activated β-lactams.

Subsequent conjugation of modified ACP to a fluorophore (red star) uses standard click chemistry. The resulting fluorescent ACP is used as the fluorescence donor in the current study.

As discussed in earlier chapters, we have recently developed a series of β-lactone and β-lactam probes capable of directly acylating the Ppant-thiol of *holo*-ACPs.[17,18] Incorporation of an alkyne functionality provides a chemical handle for conjugation with fluorophores (**Figure 22.**)**.**The thioester-ACP resulting from incubation of *holo*-ACP with a propargyloxycarbonyl (Poc)-activated β-lactam was shown to be competitive with traditional *N*-acetylcysteamine (SNAc) thioesters for KS acylation. Based on these results, it occurred to us that fluorescent thioester-ACPs, derived from an alkyne-bearing β-lactam, coupled with acyl-SNAc compounds, could provide a straightforward means of examining KS selectivity in whole PKS modules. More specifically, the degree of KS acylation for a given SNAc substrate may be assayed by introducing a fluorescent ACP after incubation of KS and substrate and monitoring fluorescence transfer *via* gel electrophoresis. The amount of fluorescence transfer would therefore indicate to what extent the SNAc compound was accepted by the KS domain (**Figure 23**.). High KS-loading would block subsequent fluorescence transfer from ACP, and thus produce little to no fluorescent

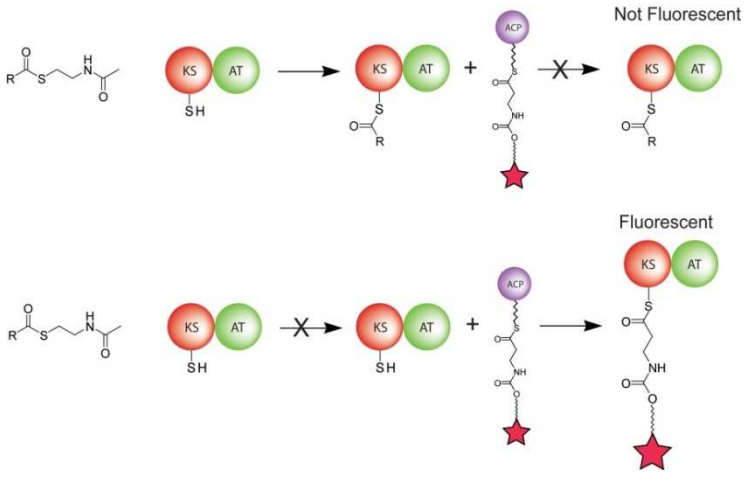

Figure 23. Schematic of mechanism-based, fluorescence transfer assay for KS-selectivity

A given KS domain is incubated with acyl-SNAc followed by addition of fluorescently labeled ACP. The amount of fluorescence transferred from the ACP to the KS active site provides a direct readout of the substrate tolerance for each acyl-SNAc examined. KS = ketosynthase, ACP = acyl carrier protein, AT = acyltransferase, SNAc = *N*-acetylcysteimine.

signal in the gel. Alternatively, insufficient KS-acylation with a SNAc compound would leave most KS active sites free to accept the fluorescent substrate from ACP resulting in a bright band. This technique would afford a direct measure of KS selectivity while circumventing the limitations of mass spectrometry methods described above. Most importantly, the use of mechanism-based fluorescence transfer from acyl-ACPs ensures that any fluorescence observed on the acceptor module results solely from the KS active site and not from competing, nucleophilic surface residues within the complex protein system.

We expect the straightforward nature of this method to permit all research labs to explore key features of polyketide construction. Studies ranging in scope from basic KS selectivity to more complex ACP/KS complementarity for designing hybrid systems will be greatly simplified, ultimately leading to significant advances in PKS engineering. Herein, is described the initial work toward designing a mechanism-based, fluorescence transfer method for examining substrate selectivity in PKS assemblies.

4.2. Results and discussion

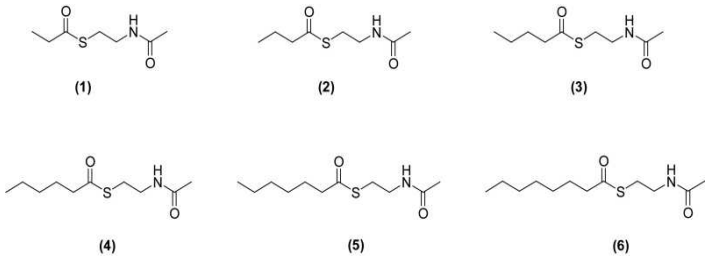

Figure 24. Panel of SNAc thioesters to probe chain-length selectivity

Our previous work demonstrated that 6-deoxyerythronolide B synthase (DEBS) module 2 ACP (ACP2) could efficiently transfer a β-lactam-derived fluorescent probe to the KS active site of the DEBS module 6 KSAT[19] didomain (KSAT6).[18] Since a careful choice of the donor ACP is critical for success using this technique, we decided to begin with this ACP/KS pair for proof of

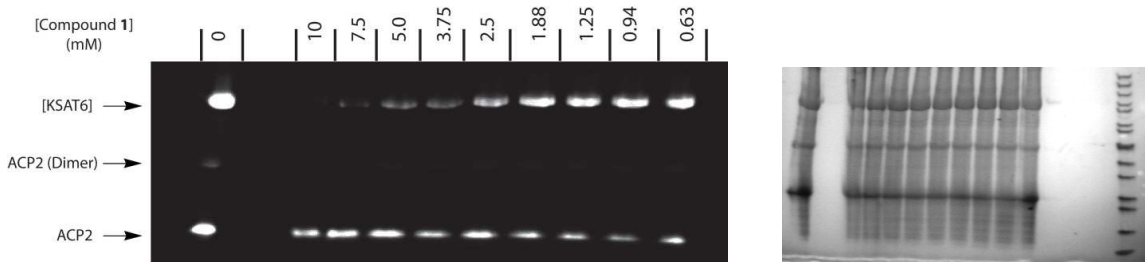

Figure 26. Fluorescence transfer from ACP2 to KSAT6 with variable conc. of **1**

(Left) Lack of fluorescence indicates near quantitative acylation of the KS active site with compound **1**. Bright bands indicated little to no KS loading. Lane 1 corresponds to the positive control where no compound **1** is added. Numbers at the top indicate the concentration of compound **1** added to KSAT6. Right figure is full PAGE gel stain.

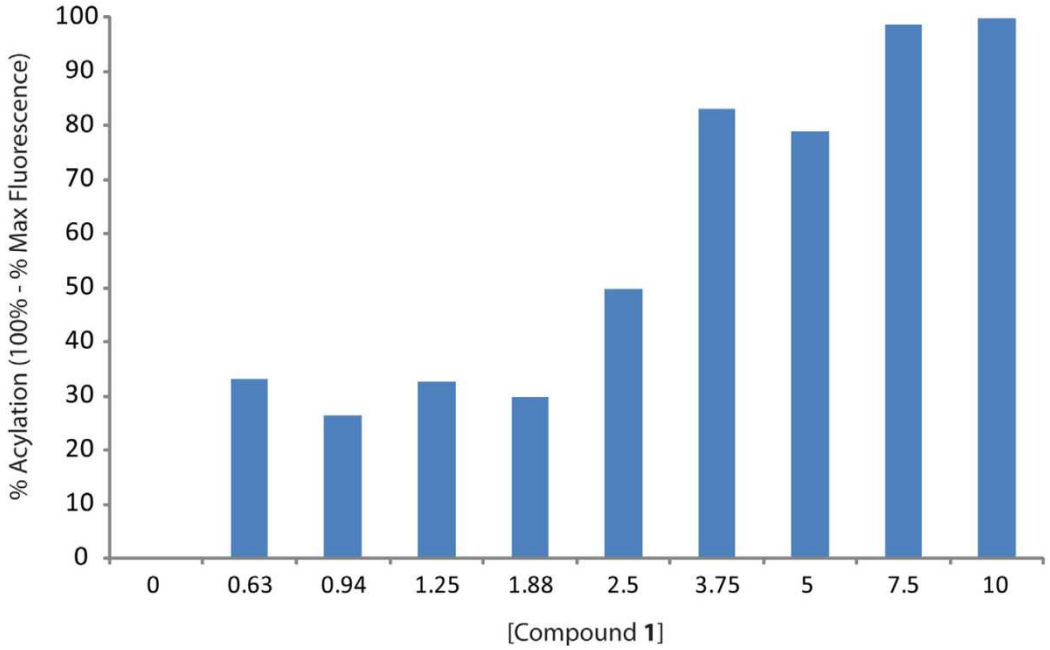

Figure 25. Fluorescence intensity measurements for KS acylation

Fluorescence intensities for the above gel were obtained via fluorescence analysis using the ImageJ software. % KS acylation for each concentration of compound **1** assayed was calculated by subtracting the % of maximum fluorescence (determined by comparison to the control lane) for each band from 100%. A plot of % Acylation versus compound **1** concentration is shown below.

principle studies. We had previously shown that fluorescence transfer from ACP2 to KSAT6 was exclusive at the active-site as no transfer occurred in the presence of the KS active site

inhibitor cerulenin, and was complete in as little as 30 min. Encouraged by the impressive speed and specificity of this process, we commenced the development of a mechanism-based fluorescence transfer assay capable of directly probing the influence of substrate structure (*i.e.* chain length, sterics, and electronics) on KS loading.

Based on the knowledge of biosynthestic process of DEBS, we made some initial hypothesis to start with. Firstly, we expected that longer chain substrates would be preferred based on the structure for the natural hexaketide recognized by module 6 KS. In addition, module 5 incorporates a methylmalonyl-extender unit which, following elongation, is passed to module 6. As a result, we further hypothesized that α-branched thioesters may be particularly

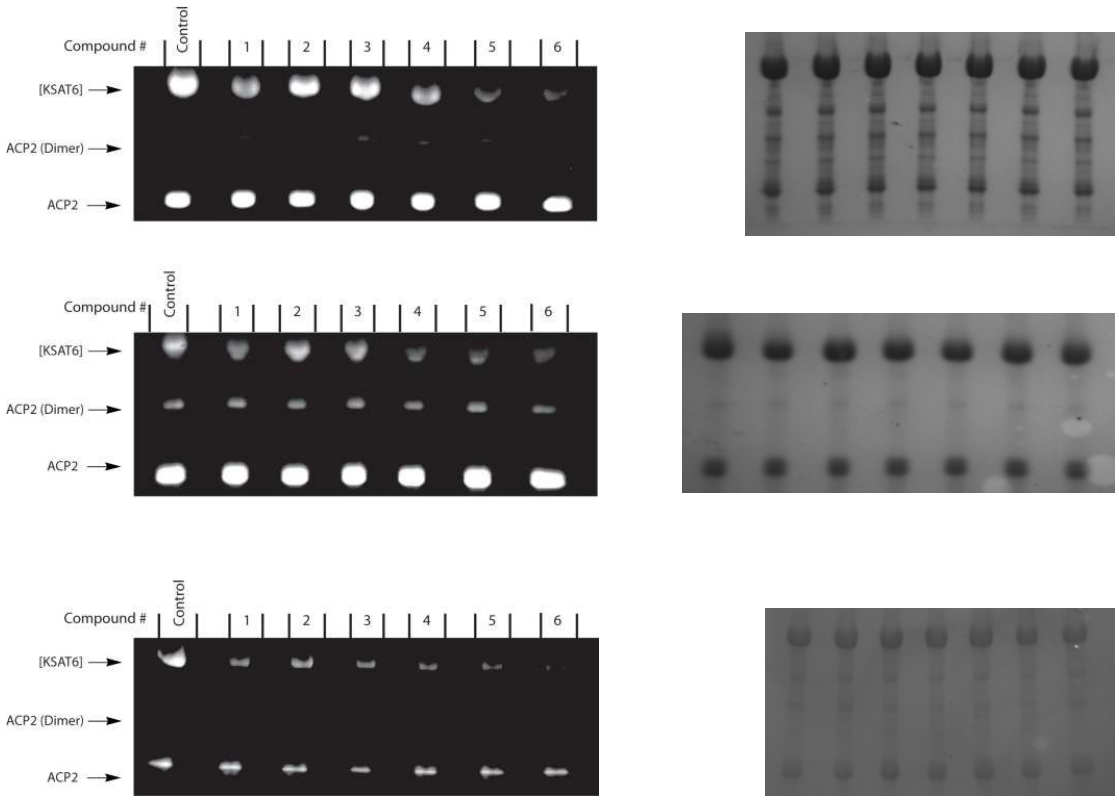

Figure 27. Fluoscence transfer from ACP2 to KSAT6 with compounds **1-6**

KSAT6 is pretreated with compounds **1**-**6**. (Left) Lack of fluorescence indicates near quantitative acylation of the KS active site with compound **1**. Bright bands indicated little to no KS loading. Lane 1 corresponds to the positive control where no acyl-SNac is added. Numbers at the top indicate which compound KS was treated with prior to addition of fluorescent ACP.

well-suited for acylating the KS domain. Our fluorescence transfer method would allow us to readily observe these tendencies and, more importantly, provide high-resolution information as to their limits.

Since PKS intermediates are of variable chain lengths at different stages of processing, we wished to begin by examining this simple aspect of chain length preferences for KSAT6. A panel of SNAc thioesters derived from straight-chain carboxylates was prepared (**Figure. 24**). Before we could begin, however, it was necessary to determine an appropriate acyl-SNAc concentration where subtle differences in KS-loading could be readily observed. Fluorescently labeled ACP was prepared as previously described by reacting *holo*-ACP2 with *N*-propargyloxycarbonyl-β-lactam followed by Cu-catalyzed [3 + 2] cycloaddition with rhodamine azide (see **Figure. 21**).[20–22] Excess click reagents were removed *via* filtration to avoid any complications during the assay. To determine the optimal substrate concentration for KS-selectivity studies, compound **1** was introduced to KSAT6 at concentrations ranging from 630 μM to 10 mM. After 1 h incubation, a slight excess, relative to KSAT6, of fluorescent ACP2 was added for 30 min and the protein components were subsequently separated and analyzed by SDS-PAGE. From these data, it was determined that the inflection point between fully loaded and unloaded KS corresponded to a substrate concentration of roughly 2.5 mM for compound **1** (**Figure 25**.). The fluorescence intensities were analyzed using ImageJ software which shows trends consistent with the visual results. This analysis suggests that the visual read-out of this method is at least pseudo-quantitative and can be used with good confidence for comparison of different substrates.

The variable chain length SNAc compounds (**1–6**) were subjected to the same analysis as above using 2.5 mM of each. To our satisfaction, a clear preference for longer chain substrates was observed (**Figure 27**.). Given that DEBS module 6 KS normally accepts a hexaketide intermediate from module 5, these results are consistent with what might be expected of

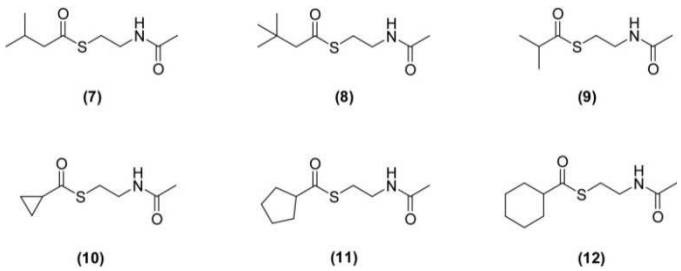

Figure 28. Panel of SNAc thioesters to probe α- and β-branching selectivity

modules further down the biosynthetic assembly line. Most importantly, the observed trend was reproducible over multiple, separate runs (**Figure. 27**.). It should be noted that previous attempts on our part to monitor KS-loading with compounds **5** and **6** *via* tandem proteolysis/mass spectrometry were unsuccessful most likely due to precipitation of the

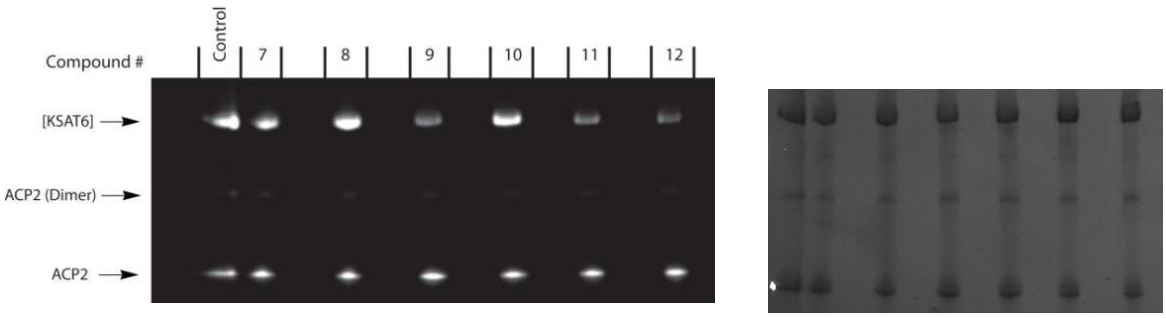

Figure 29. Fluorescence transfer from ACP2 to KSAT6 with **7-12**

(Left) Lack of fluorescence indicates near quantitative acylation of the KS active site with compound **1**. Bright bands indicated little to no KS loading. Lane 1 corresponds to the positive control where no acyl-SNac is added. Numbers at the top indicate which compound KS was treated with prior to addition of fluorescent ACP. (Right) PAGE full gel stain

acylated KS active site fragment.

We were highly encouraged from these results and were excited to examine the effects of more complex structural components on KS-loading. As PKS enzymes often display the ability to identify and distinguish α- and β-position substitutions, we were interested in uncovering any substrate preferences for branched alkyl-SNAc compounds. A small panel of these compounds

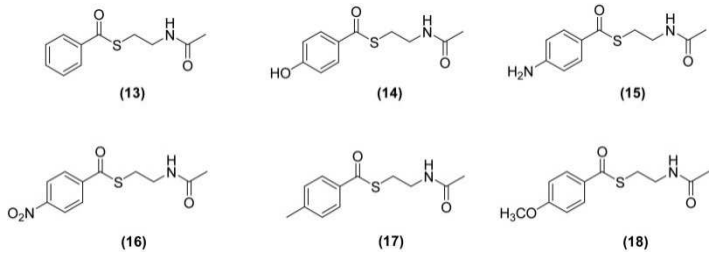

Figure 30. Panel of SNAc thioesters tp probe *p*-substituted aryl groups

was prepared with variable branching at both the α- and β-positions of the carbonyl (**Figure. 28).** Since the natural polyketide intermediate accepted by DEBS module 6 KS harbors an α-methyl group, it is not surprising that compounds **9**, **11**, and **12** appear to load KSAT6 quite efficiently while compounds **7** and **8** are not as effective (**Figure. 29**). The cyclopropyl-containing compound **10**, however, is very interesting. Despite the presence of an α-branch, this substrate clearly behaves more like those without α-substituents. It is our thought that this may arise from the extreme cyclopropane bond angles which might alter the side chain geometry to the point where the KS active site does not recognize any branching. Regardless of its origins, this is a rather remarkable response to very subtle differences in structure between isopropyl and cyclopropyl groups.

Having analyzed structural aspects, we wanted to extend the idea to examine electronic aspects of KS-acylation. Using a phenyl ring as a starting template we wanted to prepare a panel aromatic compounds bearing various electron withdrawing and donating groups to vary the extent of activation and deactivation, respectively. A set of acyl-SNAc compounds was prepared with an assortment of *p*-substituents for comparison to the parent phenyl ring of compound **13(Figure 30).** From the data, it appears that all *p*-substituents confer at least a slight reactive advantage relative to the unsubstituted phenyl ring (**Figure 31**). To our delight, the nitrophenyl compound **16** is most reactive as might be expected from the increased carbonyl electrophilicity offered by the electron withdrawing nature of the nitro group. Clearly, the electronics of the thioester play a significant role in KS acylation, but without structural information regarding the active site binding interactions of each of these substrates, it is difficult to speculate further. Taken together with the results from the chain length and branching studies, this method is capable of generating a wealth of information regarding KS selectivity in short order with minimal cost and synthetic manipulation. However, to showcase its maximum value for examining substrate tolerance in polyketide biosynthesis, we felt it

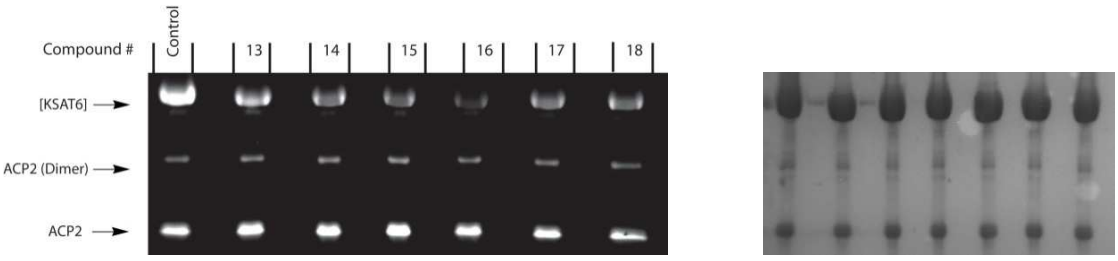

Figure 31. Fluorescence transfer from ACP2 to KSAT6 with **13-18**

(Left) Lack of fluorescence indicates near quantitative acylation of the KS active site with compound **1**. Bright bands indicated little to no KS loading. Lane 1 corresponds to the positive control where no acyl-SNac is added. Numbers at the top indicate which compound KS was treated with prior to addition of fluorescent ACP. (Right) PAGE full gel stain.

appropriate to move away from didomains in favor of full PKS modules.

To extend the scope of this method to the next level of complexity we wished to apply it to full module. Keeping consistent with previous data sets, we reasoned that DEBS module 6 would be the most appropriate target for this study. This module contains a ketoreductase, acyl carrier protein, and thioesterase domain in addition to the KS and AT present in the didomain construct. The *apo* form of ACP was expressed and used for this study to avoid any competition with the native ACP of this module. In addition, the potential for fluorescence transfer from ACP2 to the thioesterase domain, positioned at the C-terminus of DEBS module 6, prompted us

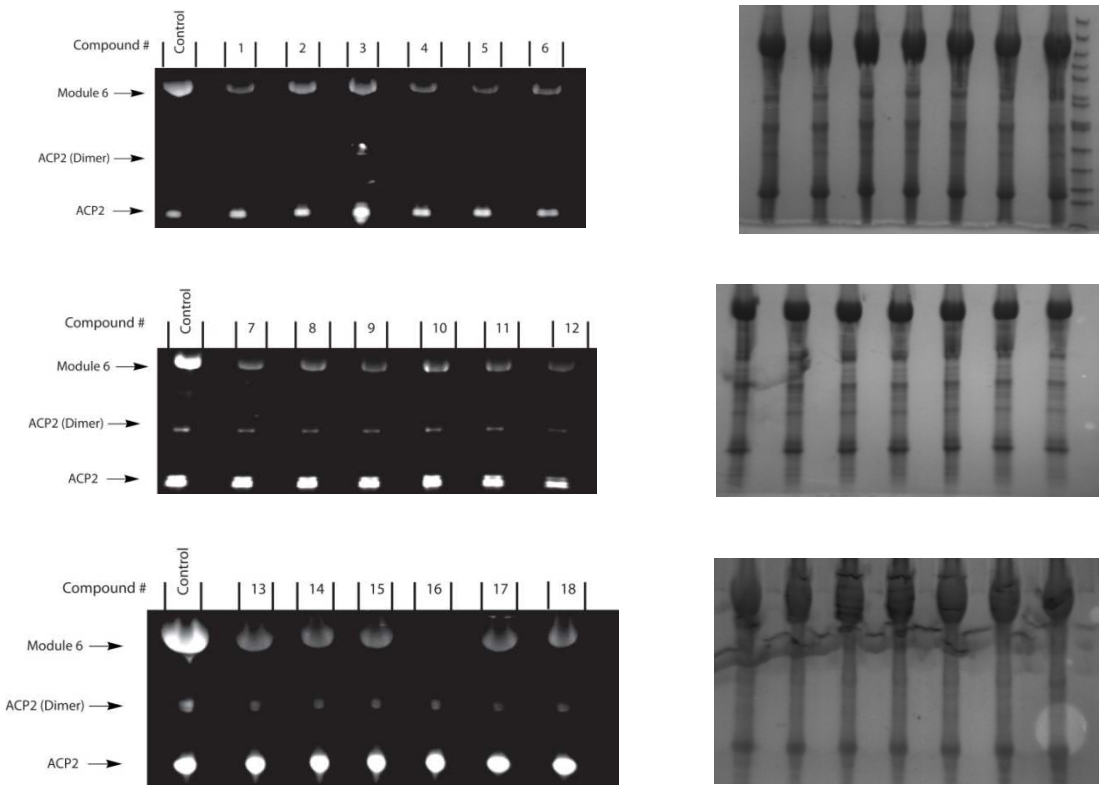

Figure 32. Fluorescence transfer from ACP2 to *apo*-Mod6 with **1-18**

(Left) Lack of fluorescence indicates near quantitative acylation of the KS active site with compound **1**. Bright bands indicated little to no KS loading. Lane 1 corresponds to the positive control where no acyl-SNac is added. Numbers at the top of each gel indicate which compound module 6 was treated with prior to addition of fluorescent ACP. Overall trends in each gel are consistent with what was observed for KSAT6 above indicating that the presence of DH, ER, KR, and ACP domains does not significantly alter KS-selectivity. (Right) PAGE full gel stains.

to omit this entity from the construct used in this study. With the optimized full module in hand, the same 18 probes used to probe KSAT6 selectivity were applied.

Overall, it was observed that the general trends observed for KS-selectivity in the didomain precisely tracked those for the intact module although the contrast between "good" and "bad" substrates is slightly diminished (**Figure 32.**). The preference for both longer chain and α-branched substrates is again evident and the remarkable distinction between isopropyl and cyclopropyl groups appears consistent. The role of electronics in KS-acylations is demonstrated by the observed preference for the nitrophenyl-containing compound 16 over all other aryl-SNAc substrates. Taken together, these results offer a clear picture of the substrate scope tolerated by this KS in two unique environments. The fact that the observed trends are consistent between the didomain and intact module provides credence to the use of "broken modules" for this kind of analysis and for its future applications.

4.3. Conclusion

We describe the development of a mechanism-based, fluorescence transfer assay for substrate selectivity in KS domains. It is a great application of the technique of ACP-acylation with β-lactams generating functional fluorescently labeled intermediates, as described in Chapter 3. This technique in its current form, provides a simple, qualitative output without the need for expensive reagents or equipment. The KS-selectivity studies are an example of the vast and diverse number of applications to which this method can be extended, including: (1) further examination of substrate attributes (*i.e.* stereochemistry, heteroatom incorporation, *etc.*), (2) KS/ACP complementarity, (3) determining the impact of substrate binding pocket mutations within KS domains on substrate tolerance, and (4) assessing the influence of active-site

mutagenesis on downstream KS substrate selectivity. The work described here properly lays the necessary groundwork toward each of these endeavors. In all, we expect this method to significantly aid in the study of new and existing PKS systems leading to improved understanding of how these extraordinary biosynthetic machines function.

4.4. Experimental Details

Protein expression

ACP2 was expressed from pNW06,[8] ACP3 was expressed from pVYA05.[19] [KS6AT6] was expressed from pAYC11.[12] *apo*-ACPs were harvested from *E. coli* BL-21 and *holo*-ACPs were harvested from *E. coli* BAP-1.[25] pNW06 and pVYA05 contain kanamycin resistant vectors.

Cloning (pTL-DM6)

Cloning of DEBS-Mod 6 (without TE domain) was done by PCR on pRSG54 (a gift from Professor Khosla, which encodes for DEBS Mod 6+TE). Using the primer DEBS-M6-NdeI-F1: TTTTTTCATATGGACCCGATCGCGATCGTCGGCATG, and DEBS-M6-HindIII-R1: AAAAAAAAGCTTGAGCTGCTGTCCTATGTGGTCGGC, PCR was performed in a 50 µL reaction mixture containing: 1 × Phusion GC buffer, 0.2 mM dNTP, 0.2 mM $MgCl_2$, 15% Glycerol, 1 µM of each primer, 1 U Phusion Hot Start II DNA polymerase (Thermo scientific), and approximately 300 ng of pRSG54 DNA. PCR amplicon of 4215 bp was obtained after 35 cycles PCR amplification. The DNA amplicons were excised and purified by using QIAquick Gel Extraction Kit (QIAGEN, Germany) and then directly subjected to double restriction enzyme digestion of NdeI and HindIII. The digested product was ligated to pre-digested pET-21b to obtain pTL-DM6.

Protein purification and isolation

Bacteria were grown in 1 L shake cultures of LB-antibiotic media at 37 °C in an incubating shaker until the OD600 was between 0.6 and 0.8. Over expression was induced with 200 µL of 1 M IPTG (per liter of culture) and carried out at 18 °C for 18 h. After this point all work was carried out at 4 °C. Cells were pelleted by spinning at 5000 RPM for 10 min and resuspended in 50 mL of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium Na_3VO_4, 1 µg mL^{-1} leupeptin, pH 7.5). Cells were lysed using an ultrasonic converter. The lysate was pelleted at 10 000 rpm for 60 min. The supernatant was equilibrated with 3 mL of Ni-NTA slurry (per liter of culture) for 60 min by stirring with a PTFE-coated stir bar at minimal speed. The mixture was then poured into a fritted column and the supernatant eluted. The resin bed was washed with two 15 mL portions of wash buffer (50 mM phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0), and eluted with 3 mL (per liter of culture) of elution buffer (50 mM phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0). The purified protein was loaded into a 3 kDa NMW centrifugal concentrator and diluted to 15 mL with storage buffer (100 mM Tris, 1 mM EDTA, 1 mM dithioerythritol, 10% glycerol pH 8) and spun until the final volume was ≤500 µL. Dilution and filtration was repeated a total of three times. Protein concentration was determined by Bradford assay with a BSA standard curve. Proteins were flash frozen and stored at −80 °C until use.

Fluorescence transfer assay

Unless otherwise stated, phosphate buffer refers to 100 mM, pH 7.0 phosphate. ACP2 (25 µM total protein) was equilibrated at ambient temperature for 15 min in phosphate buffer containing 2.5 mM TCEP. Poc β-lactam (10× with respect to total ACP concentration) was added and the mixture was equilibrated at ambient temperature for 45 min. Rhodamine Azide (2× with respect to Poc β-lactam), sodium ascorbate (1 mM), and copper(II) sulfate (1 mM) were added

to samples which had been labeled with **6**. The reaction was performed at ambient temperature for 45 min. To remove excess Poc β-lactam and Rhodamine Azide, the mixture was loaded into a 3 kDa NMW concentrator and the volume reduced to 100 µL by spinning in a centrifuge cooled to 4 °C. The mixture was diluted to 500 µL with phosphate buffer, and then concentrated to 100 µL again. This process was repeated a total of 5 times. Protein was removed from the concentrator by inverting it and spinning. For transfer of the fluorescent product, [KSAT6] or the full module (25 µM) was pre-treated with respective SNAc (2.5 mM) derivatives for 1 h. This mixture was introduced to filtered acyl-ACP2 and incubated for 30 min. The [KS6AT6] and *apo*-Mod6 control was executed under the same acylation and click conditions as in other samples but without the SNAc derivative and therefore represents full acyl transfer.

Chromophore attachment

The reaction was carried out at 25 µL, final concentrations reported. Rhodamine Azide, (2× with respect to Poc β-lactam), sodium ascorbate (1 mM), and copper(II) sulfate (1 mM) were added to samples which had been labeled with **6**. The reaction was performed at ambient temperature for 45 min.

Gel assay

Labeled samples were diluted to 20 µL with gel-loading buffer. Proteins were separated using a 4–20% gradient HEPES-PAGE gel (100 V, 50 mA, 70 min). Gels were developed in 10% acetic acid to visualize Rhodamine Azide. Labeled proteins were imaged on a UV-transilluminator. Total protein was stained using Coomassie stain.

Prop-2-yn-1-yl 2-oxoazetidine-1-carboxylate: 2-Azetidinone (107 mg, 1.5 mmol) was dissolved in THF (7 mL) at −78 °C. LiHDMS (330 mg, 2 mmol) dissolved in THF (2 mL) was added in portions over 10 min and the reaction mixture was stirred for 30 min. Propargyl chloroformate (181 mg, 1.5 mmol) dissolved in THF (2 mL) was added in portions over 10 min and the reaction mixture

was stirred at −78 °C for 2 h then allowed to warm to ambient temperature. The reaction mixture was diluted with water and extracted with DCM (50 mL × 3). The combined organic layers were washed with brine and dried over Na_2SO_4. Solvent was removed by rotary evaporation. Purification of crude product by flash chromatography with 95 : 5 DCM–Methanol yields (106 mg, 0.69 mmol) 46% of product as pale yellow solid.

Sulforhodamine-B azide: 3-Azido-1-aminopropane[23] (22 mg, 0.22 mmol) and triethylamine (51 mg, 0.5 mmol) were dissolved in 1 mL of 5 : 1 DCM–DMF and cooled to 0 °C. Sulforhodamine B sulfonyl chloride [mixture of *ortho*- and *para*-sulfonyl chloride isomers] (115 mg, 0.20 mmol) was added portion-wise over *ca.* 30 min and stirred overnight at room temperature. Solvent was removed *in vacuo*, and the product was purified by flash chromatography over silica gel with 90 : 5 : 5 DCM–Acetonitrile–Methanol mobile phase. The *ortho* isomer was distinguished by its reversible color change in pH 9.0 buffer.[24] *Para* isomer: (22 mg 0.034 mmol) 17%, *ortho* isomer (18 mg, 0.028 mmol) 14%. Both obtained as a red solid with a metallic green luster. The *ortho* isomer was found to perform best in the click reaction and was utilized for labeling experiments.

General method for preparation of all *N*-acetylcysteamine (SNAc) thioester derivatives

To a solution of triethylamine (2.80 mmol) in dichloromethane (10 mL) was added the appropriate acid (1.40 mmol), (3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.40 mmol), 1-hydroxybenzotriazole (HOBt) (1.40 mmol) and *N*-acetylcysteamine (SNAc) (1.35 mmol) under argon. The reaction mixture was stirred overnight. The organic layer was washed with saturated $NaHCO_3$ solution, 0.1 N HCl solution and brine. It was then dried over anhydrous sodium sulfate, concentrated under vacuum, and purified by flash column to provide the final product in pure form.

4.5. References

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CHAPTER 5

EXAMINING KS SELECTIVITY IN FLUVIRUCIN B_1 SYNTHASE

5.1 Introduction

Over the past several decades, researchers have devised numerous methods for manipulating PKS processes in an effort to diversify product structure and, ultimately, biological activity.[1-5] In the effort to capture the true engineering potential of PKSs, numerous techniques have been explored for separation, fusion, and rearrangement of component enzymes.

The domain swapping technique involves physically replacing an individual PKS enzyme with one from another synthase. Most commonly used for AT domains, the key to effective domain swapping is comprehensive knowledge of domain boundaries such that the surrounding enzymes are unaffected. The primary downside to this procedure is that heterologous domains often behave differently in their new environment. The domain addition technique is generally employed when an increase in enzyme activity is desired. Many attempts have been made to incorporate KR, DH, and ER domains into modules where they are absent but the most effective strategy for domain addition involves replacing the entire module with a heterologous one bearing the appropriate tailoring domains. Although some success has been realized with these techniques, decreased yields of engineered products have limited their scope and efficacy. In both cases, introduction of heterologous enzyme components often leads to disruption of three-dimensional structure and causes protein communication issues between native and heterologous components, often impeding the flow of intermediates within and between modules. These factors together contribute to the issue of decreased yields.[6-11]

The simplest technique of all is the domain deletion/inactivation, functionally the opposite of domain addition. The activity of any module containing at least one tailoring domain (KR, DH, or ER) can be altered via mutation of active site residues or removal of the entire enzyme. It has been shown, however, that mutation (inactivation) provides significantly higher product yields compared to removal (deletion), owing largely to the fact that the former method maintains the native three-dimensional structure of the engineered module. This observation together with the domain addition and swapping data, form the initial motivation and basis of our hypothesis. We were convinced that disruption of PKS structure and recognition motifs plays a significant role in diminished product yields.

For modular polyketide synthases this means unearthing assemblies which bear the full complement of tailoring domains (KR, DH, and ER), similar to mammalian FAS, in most, if not all, active modules. In contrast to current methods, carefully executed mutagenesis of key native active site residues should result in all possible β-functionalities. The key advantage will be the ability of leaving the native protein-protein interactions intact (**Figure 33**). Therefore, we were particularly interested in PKSs that produce largely unfunctionalized polyketides (i.e. methylenes at most β -positions) and may provide optimal engineering potential.

Our lab has recently identified the gene cluster for a modular PKS responsible for the production of fluvirucin B_1 in *Actinomadura vulgaris* (**Figure 34**.).[12-14]Fluvirucin B_1 is a 14-membered macrolactam with moderate to good antifungal and antiviral activities. It is an interesting PKS system with many unique biosynthesis attributes. It contains 5 modules of which 4 contain the full set of PKS tailoring domains. According to our hypothesis, it is an ideal system for engineering strategy of single domain inactivation via site-directed mutagenesis.

We were motivated to explore the biosynthetic origin of fluvirucin B_1 with the ultimate goal of providing a platform for polyketide engineering that circumvented the need for

incorporation of heterologous domains to achieve maximal product diversity. By using the strategy of active-site mutagenesis, we can inactivate one enzyme at a time to access all the β - functionalities possible **(Figure 33.**). The fluvirucin polyketide synthase offers nearly double the product scope of the most commonly used assembly, 6-deoxyerythronolide B synthase, due to the significantly increased number of ketoreductase, dehydratase, and enoyl reductase domains within the cluster. By using this approach, there is a potential to produce large library of macrolactam variants. Such a system provides an ideal platform for conducting various kinds of PKS investigations with the current set of β-lactam based molecular tools that we have successfully developed.

The lack of additional ring functionalities caught our interest as we hypothesized that nearly all active modules should contain the FAS-like domain organization where KR, DH, and ER are all present. That is another interesting feature of this PKS system in terms of resemblance to FASs. If one were to consider PKS systems as evolutionarily connected to FAS, the fluvirucin synthase may represent an early link between the two. These investigations of their biosynthetic mechanisms can help shed some light on this aspect as well.

Role of substrate selectivity

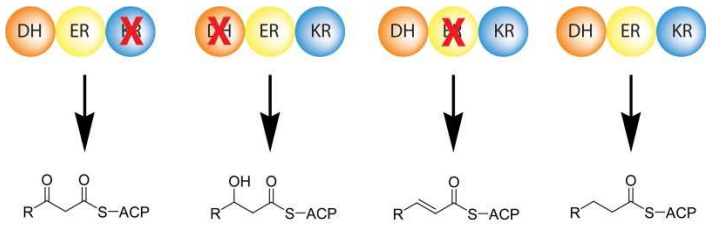


Figure 33. Schematic for mutational inactivation strategy of fatty acid-like PKS modules
Novel compound production by designing alterations in chemical structure at the genetic level.

Flow of intermediates along a PKS system heavily relies on communication between neighboring enzymes). Many groups have focused on determining the associated recognition motifs that govern these interactions.[15-17] However, we were interested in obtaining product diversity without reinventing enzyme architecture and work with what nature has already provided. To do so, a strategy where product diversification is solely the result of domain inactivation must be implemented. Without disturbing the protein-protein interactions by leaving all recognition interfaces in their natural forms, the problem of producing and shuttling intermediates along the synthase will now be reduced to one of substrate selectivity. Above all, it will rely on the ability of downstream KS to accept modified intermediate substrate handed down by an upstream mutant module.

Instead of using radiolabelled substrates that would require elongation and cyclization events or struggle with LC/MS to tease out differences in small molecular masses of the highly similar intermediates, it occurred to us that the mechanism-based fluorescence transfer assay to investigate KS selectivity as described in Chapter 4 could be of great assistance for this purpose [18]. Firstly, we prepare a library of potential substrates with β-functional modifications for each of the KSs and use them to assay KS compatibility for these novel β-carbon analogues that they would encounter during biosynthesis in a modified, mutant module. In this manner, this assay can be used as a prototype that mimics substrate transfer and intermediate flow to test the effectiveness of the mutagenesis in the module. In addition to delivering improved yields of engineered products, such a system will, for the first time, provide a systematic means of assessing the relative contribution of substrate selectivity to the flow of structures from one module to the next in full-length PKS.

The ultimate test of this methodology is to combine high yielding single mutants to build increased complexity and diversity in polyketide structure by using a multiple-plasmid system to

incorporate more than one mutation. In which case, the KS-selectivity assay can be employed to gain insight regarding behavior of downstream enzyme to a particular upstream structural modification caused by a mutation in an intact PKS and consequently help in logical designing of these complex assemblies. In this manner, our primarily goal is to use this KS-selectivity assay as a tool to lay the groundwork to ensure success of the product diversification strategies. But in turn, this will provide a wealth of information regarding influence of upstream modifications on downstream enzyme activity and help improve our understanding of the biosynthetic machinery and its working.

5.2. Results and discussions

From the core structure of fluvirucin B_1, we hypothesized that the producing PKS would consist of five extender modules assuming that a β-alanine derivative is used as a starter unit in the process. The sole hydroxyl group would therefore arise from a module harboring only a KR domain while all other extender modules would contain the full complement of KR, DH, and ER domains (**Figure 34.**). Based on the positions of macrolactam ring substituents, we expected that: (1) the first and last modules contained ethylmalonyl-specific AT domains, (2) the second and fourth modules incorporated malonyl groups, and (3) the third module utilized methylmalonate (**Figure 34.**). Finally, ring closure was most likely achieved via a C-terminal thioesterase (TE) domain as is the case with similar macrocyclic polyketides.[19-21] To test these hypotheses and determine the precise arrangement of enzymes within the assembly, we set out to identify and characterize the fluvirucin B1 biosynthetic gene cluster.

To do so, the producing organism, Actinomadura vulgaris, was cultured following published procedures.[13-15] Genomic DNA was isolated and sequenced affording 436,311

overlapping sequence fragments. These sequences were partially assembled resulting in 444 consensus sequences ranging in size from 5000 to 170,000 base pairs. The relatively large size of PKS constructs allowed us to quickly identify potential hits by searching each assembled sequence for open reading frames of at least 4000 base pairs. Our search identified several PKS gene clusters, one of which contained the expected size and module composition of the proposed fluvirucin PKS.

Three modular PKS genes, flu A-C, were found to contain an arrangement and composition of domains consistent with the expected fluvirucin PKS assembly[22] (**Figure 34**.). FluA contains modules 1 and 2 of the fluvirucin B_1 assembly. As expected, module 1 has the full compliment of tailoring domains (KR, DH, and ER) while module 2 possesses only a KR domain putatively leading to the sole hydroxyl group on the macrolactam ring. A single loading ACP is found at the N-terminus of FluA similar to the vicenistatin PKS which utilizes a β -methyl- β -alanine starter unit.[23] FluB contains modules 3 and 4 of the fluvirucin B_1 PKS. Both modules contain KR, DH, and ER domains as would be predicted from the fluvirucin core structure. Finally, FluC consists of module 5 and a C-terminal TE domain. Module 5 again has all three tailoring domains consistent with the lack of functionality at the corresponding macrolactam ring position.

Fluvirucin B_1 PKS Genes

As predicted, the fluvirucin B_1 polyketide synthase consists of 5 extender modules flanked by an N-terminal loading ACP and C-terminal TE domain. All but one of the extender modules contains a KR, DH, and ER domain in addition to the required KS, AT, and ACP leading to the relatively unfunctionalized nature of the macrolactam product. Based on this arrangement of composition of modules, β-alanine is expected to serve as the starter unit for fluvirucin B_1 biosynthesis.

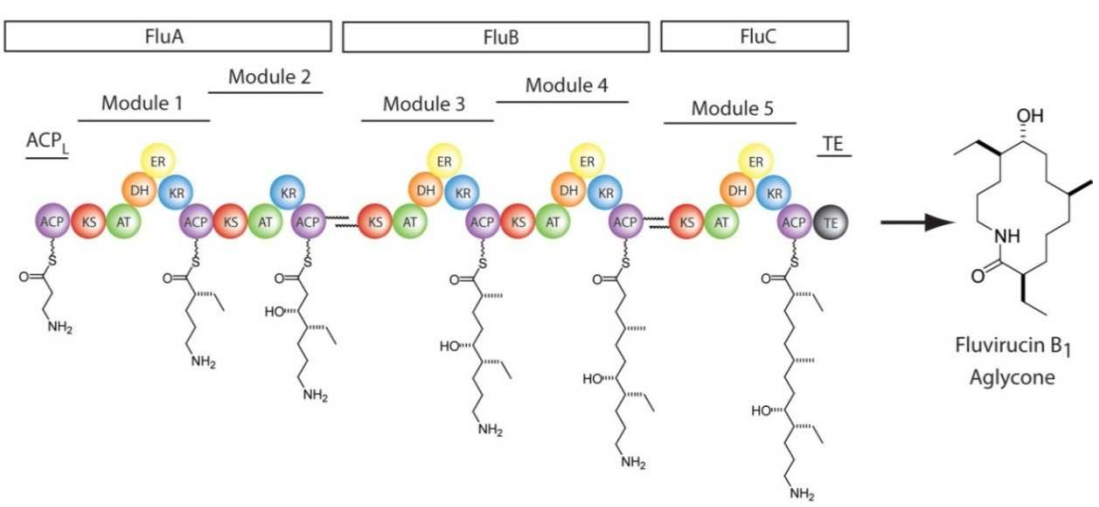


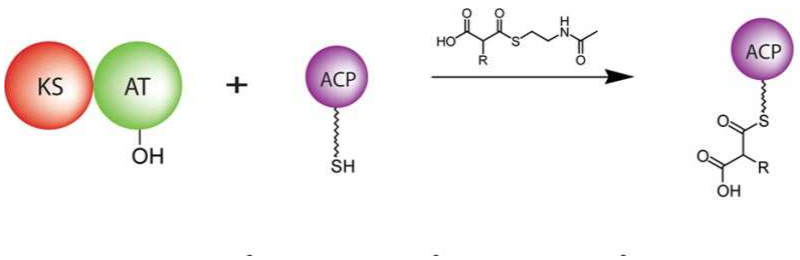
Figure 34. The putative fluvirucin B1 PKS

The assembly consists of five extender modules flanked by an N-terminal loading ACP and C-terminal thioesterase (TE) domain. FluA consists of the loading ACP, module 1 and module 2. FluB consists of modules 3 and 4. FluC consists of module 5 and the TE domain.

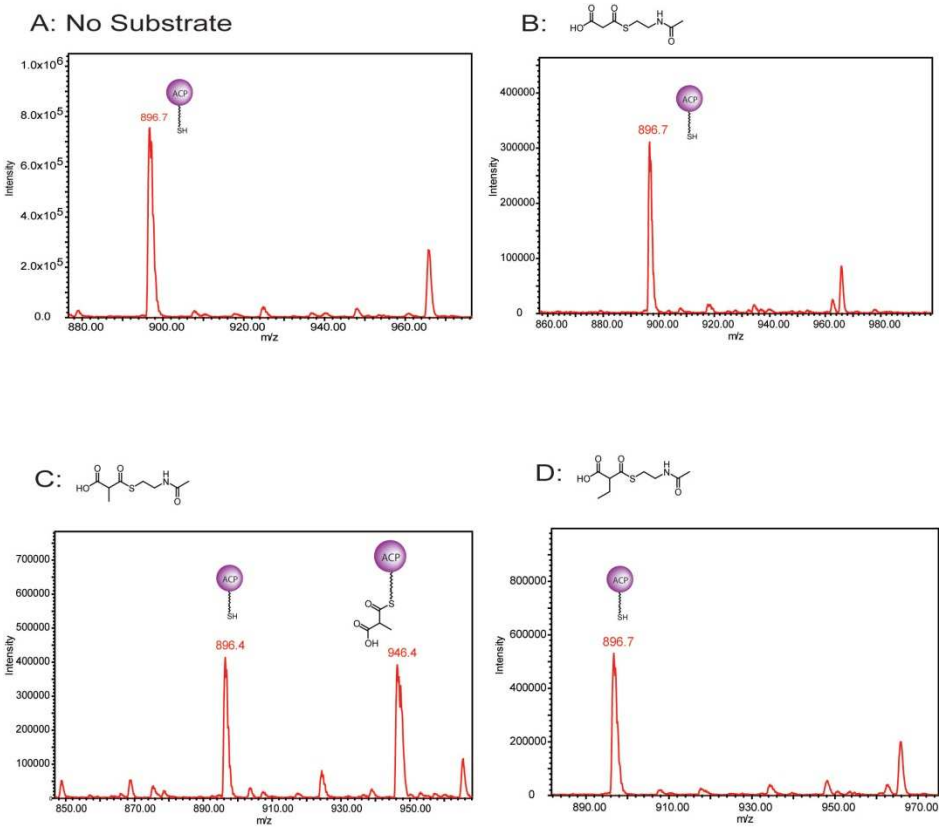
AT Selectivities

Extender unit selectivity for AT domains provides compelling evidence for any link between a given PKS assembly and its associated polyketide product. Using, the SEARCHPKS program developed by Mohanty and coworkers, probable coenzyme A substrates were determined for each of the five putative fluvirucin B_1 synthase AT domains.[24] To our delight, all of the predicted AT domain specificities were consistent with the fluvirucin B_1 core structure. Specifically, modules 2 and 4 showed high sequence similarity with malonyl-specific AT domains while module 3 was predicted to utilize methylmalonyl-CoA. Modules 1 and 5 returned a single hit for ethylmalonate specificity amidst several methylmalonyl-specific AT domains. This is most likely due to the considerably lower abundance of ethylmalonyl-specific ATs relative to methylmalonyl-specific ones rather than a true indication of relaxed specificity.

a)



b) FluAT3 substrate selectivity



c)

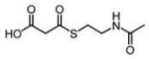
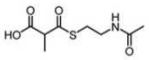
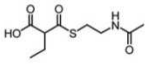
	FluAT1	FluAT3	FluAT5
	X	X	X
	X	✓	X
	✓	X	✓

Figure 35. FluAT specificity studies

a) Schematic diagram of AT substrate selectivity studies of modules 1, 3, and 5 b) representative LC-MS analysis of FluAT3 loading c) Results of AT studies, red checks indicate that the substrate shown on the left is transferred from the indicated AT to the ACP domain, whereas a black X indicates that no substrate transfer was observed. FluATX = fluvirucin AT domain from module X

In order to examine the AT specificities, the N-acetylcysteamine(SNAc) analogues of ethylmalonyl-CoA, methylmalonyl-CoA and malonyl-CoA were prepared. The ATs from Moduke 1, 3 and 5 were each tested for their ability to load their cognate ACP with these three SNAc compounds (**Figure 35**.). The ACP modification was observed by LC-MS as described previously. To our delight, as expected from the sequence similarity studies, AT1 and AT5 loaded ethlymalonyl product and AT3 loaded methylmalonyl product. These observations about the unusual substrate preferences further give credence to the enzyme identities.

Flu KS substrate selectivity

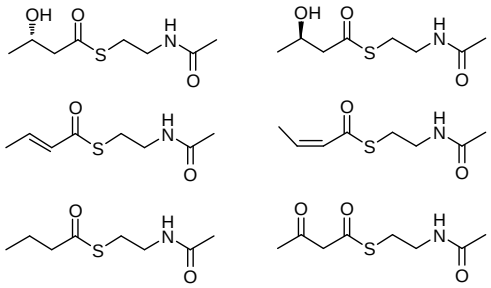


Figure 36. Panel of substrates for KS Module 3 of Fluvirucin B1 synthase

As outlined earlier, upon characterization of this unique PKS system, our aim was to test the tolerance of KSs by applying the technique developed and described in Chapters 3 and 4. This method will assess the KS selectivities towards a range of altered β-functionalities with its original substrate motif as reference. An appropriate acyl carrier protein will be chosen to generate a β-lactam based fluorescent acyl-ACP to act as a donor in this process. By tracking the extent of fluorescence transfer by the ACP to the KS active-site, we can assess the extent of KS loading by each of the test substrates. Such a straightforward analysis will provide key insight into KS preferences for different β-carbon bearing intermediates as well as shed light about the KS preferences compared to other KSs in the same PKS. All these trends and observation would

be of great value in learning about the preferences and functioning of key enzymes of this novel PKS system for which no prior information is available.

For a preliminary screen, we prepared a small panel of SNAc thioesters (**Figure 36.**). They incorporate different variations of hydroxyl, enoyl and fully reduced carbon at the beta position. This panel is a good starting point to investigate the preferences of KS for variation of functionalities at the beta position and be used further as a framework for testing the other four KSs in this PKS system. These preliminary experiments will provide some insight into the extent and contrasts of tolerance exhibited by these different KSs. To begin with, we started with the KS of module 3. We conducted a few experiments with buytryl analogues, namely: (R)and (S)-hydroxyl at β-position.

As predicted from its biosynthesis flow chart, it accepts a substrate with an (S)-hydroxyl group at its β position whereas the other stereoisomer loads the KS active site poorly resulting in substantial fluorescence transfer(**Figure 37**.). There results have been confirmed by LC-MS experiments where FluKSAT3 is incubated with both the stereoisomers and KS active site modification is analyzed by mass spectrometry (**Figure 38**.).

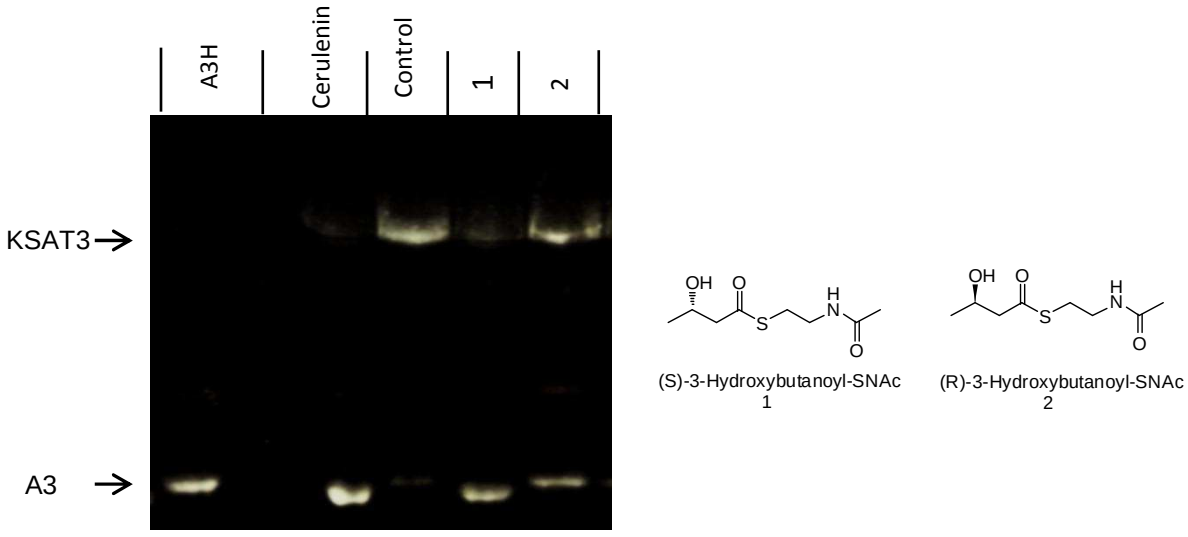


Figure 37. Fluorescence transfer from ACP3 to FluKSAT3

5.3. Conclusions

In summary, we have identified and characterized the putative PKS genes associated with fluvirucin B1 aglycone biosynthesis in A. vulgaris. The number and composition of modules as well as predicted AT specificities are consistent with the fluvirucin B1 structure. The abundance of tailoring domains within the assembly is expected to provide increased engineering potential, through straightforward active site mutagenesis. The assay developed with β-lactams derived functional acyl-ACPs for examining KS-selectivity is being applied to fluviricin KSs to understand effects of introducing novel β-functionalities on downstream KSs in terms of substrate preferences. Some preliminary work has been done with Flu KSAT3 to ensure feasibility and reproducibility and work is underway to expand the application to all the Flu KSs.

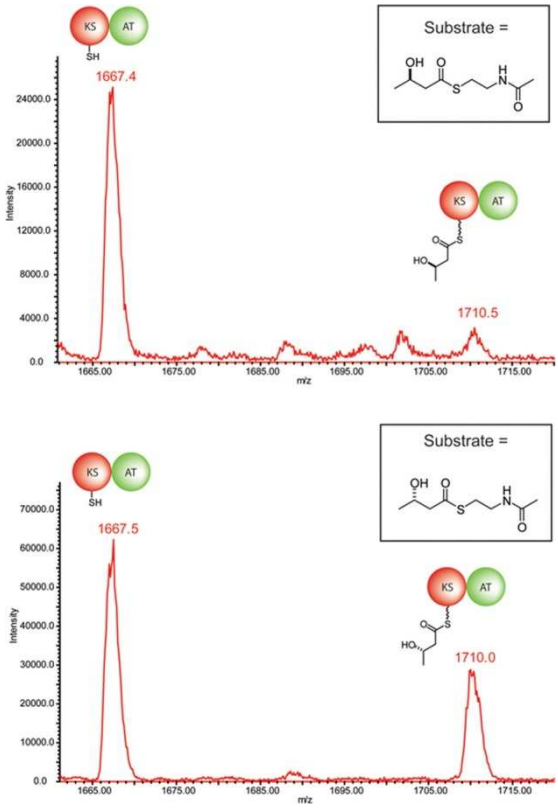


Figure 38. LC-MS loading experiments of Flu KS

LC–MS data for module 3 KS-acylation with (*R*)-3-hydroxybutyryl-SNAc (top) and (*S*)-3-hydroxybutyryl-SNAc (bottom). Only the (*S*)-isomer is accepted by module 3 KS as is expected from the fluvirucin B1 structure. Peaks are labeled with the corresponding acylated or unacylated KSAT3 didomain. All peaks are *m*/*z* = +2. Table below shows the masses for loading of KS active-site.

Table 4. KSAT3 loading with 3-hydroxy-butyric-SNAc

Protein	Probe	Mass expected(m/z, z=+2)
KSAT3	-	1667.6
KSAT3	3-hydroxy-butyric-SNAc	1710.1

5.4. Experimental Details

General method for preparation of all *N*-acetylcysteamine (SNAc) thioester derivatives

To a solution of triethylamine (2.80 mmol) in dichloromethane (10 mL) was added the appropriate acid (1.40 mmol), (3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.40 mmol), 1-hydroxybenzotriazole (HOBt) (1.40 mmol) and *N*-acetylcysteamine (SNAc) (1.35 mmol) under argon. The reaction mixture was stirred overnight. The organic layer was washed with saturated $NaHCO_3$ solution, 0.1 N HCl solution and brine. It was then dried over anhydrous sodium sulfate, concentrated under vacuum, and purified by flash column to provide the final product in pure form.

Fluorescence transfer assay

Unless otherwise stated, phosphate buffer refers to 100 mM, pH 7.0 phosphate. ACP2 (25 μM total protein) was equilibrated at ambient temperature for 15 min in phosphate buffer containing 2.5 mM TCEP. Poc β-lactam (10× with respect to total ACP concentration) was added and the mixture was equilibrated at ambient temperature for 45 min. Rhodamine Azide (2× with respect to Poc β-lactam), sodium ascorbate (1 mM), and copper(II) sulfate (1 mM) were added to samples which had been labeled with **6**. The reaction was performed at ambient temperature for 45 min. To remove excess Poc β-lactam and Rhodamine Azide, the mixture was loaded into a 3 kDa NMW concentrator and the volume reduced to 100 μL by spinning in a centrifuge cooled to 4 °C. The mixture was diluted to 500 μL with phosphate buffer, and then concentrated to 100 μL again. This process was repeated a total of 5 times. Protein was removed from the concentrator by inverting it and spinning. For transfer of the fluorescent product, [KSAT3] was pre-treated with respective SNAc (2.5 mM) derivatives for 1 h. This mixture was introduced to filtered acyl-ACP2 and incubated for 30 min. The [KSAT3] control was executed under the same

acylation and click conditions as in other samples but without the SNAc derivative and therefore represents full acyl transfer.

Chromophore attachment

The reaction was carried out at 25 µL, final concentrations reported. Rhodamine Azide, (2× with respect to Poc β-lactam), sodium ascorbate (1 mM), and copper(II) sulfate (1 mM) were added to samples which had been labeled with **6**. The reaction was performed at ambient temperature for 45 min.

Gel assay

Labeled samples were diluted to 20 µL with gel-loading buffer. Proteins were separated using a 4–20% gradient HEPES-PAGE gel (100 V, 50 mA, 70 min). Gels were developed in 10% acetic acid to visualize Rhodamine Azide. Labeled proteins were imaged on a UV-transilluminator. Total protein was stained using Coomassie stain.

ACP acylation by AT

To a mixture of ACP (50 µM) and KS-AT (2 µM) in 100mM pH 7.0 phosphate buffer (50 µL total volume) containing 2.5 mM TCEP at 4 ^{o}C was added appropriate SNAc thioester substrate (500 µM) . The mixture was incubated 4 ^{o}C for 30 min to achieve AT assisted acylation of the ACP. Sequence grade modified trypsin was added to prepare samples with final ration trypsin:ACP to be 1:10 (w/w). The mixture was incubated for 60 min at 37 ^{o}C. Digestion was quenched by addition of equal volume of 10% formic acid. Digests were stored at -80 ^{o}C until analysis.

KSAT3 loading

To a mixture of KSAT3 (25 µM) in 100 mM pH 7.0 phosphate buffer (50 µL total volume) containing 2.5 mM TCEP at 4 ^{o}C was added appropriate SNAc thioester substrate (5mM). The mixture was incubated at 4^{o}C for 60 min to achieve KS acylation. Sequence grade modified trypsin was added to prepare samples with final ratio trypsin:ACP to be 1:10 (w/w). The mixture

was incubated for 60 min at 37 ^{o}C. Digestion was quenched by addition of equal volume of 10% formic acid. Digests were stored at -80 ^{o}C until analysis.

5.5. References

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CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

Small molecule probes in the form of reactive heterocycles have been developed for direct acylation of ACP in polyketide synthases. These probes are in the form of β-lactones and β-lactams have been optimized to specifically target ACP Ppant thiol and introduce polyketide-like functionalities onto the ACP for further investigations. These reactive heterocycles have been used to introduce affinity and labeling groups onto crucial enzymes of the PKS enabling investigation of PKS biosynthesis processes.

In the first chapter, we introduced the field of polyketides and their scope and importance as therapeutics. We introduced the major traditional methods of examining PKSs and their shortcomings which have restricted the efforts in novel polyketide engineering. We also introduce our hypothesis described in detail in rest of the thesis for developing a novel approach using small molecules for examining PKS biosynthesis.

The second chapter describes use of functionalized β-lactones to achieve direct acylation of ACP. It provides a greatly simplified alternative to achieve direct, selective acylation of *holo*-ACP using readily accessible β-lactones as electrophilic partners for the PPant thiol. It offers a synthetically straightforward means of loading key PKS components with novel substrates.

The third chapter describes the introduction of novel β-lactam-based probes as versatile reagents for ACP labeling as a substantial improvement over previously described β-lactones. An versatile alkyne group is introduced to provide a handle for conjugation to fluorophores and affinity labels. The utility of these probes for mechanistic interrogation of polyketide biosynthesis is demonstrated by comparison to traditional probes.

In the fourth chapter we describe the application of β-lactam probes for designing a mechanism-based fluorescent assay for examining KS selectivity. The KS of DEBS module 6 is examined for its ability to accept a variety of simple thioesters and some remarkable observations are made. This technique demonstrates its utility by providing a simple, qualitative output without need for expensive reagents. Chapter five describes the application of the assay for examining KS selectivity to a new PKS system, Fluvirucin B1 synthase. This assay is set-up to examine selectivities of KSs from this PKS towards a panel of β-functionalities to guide the efforts of performing active-site mutagenesis in modules to produce novel polyketide products.

The fifth chapter extends the utility of this reactive heterocylce small molecule based approach to examine KS selectivity in a novel PKS system. The Fluvirucin B1 synthase has been cloned out and characterized to be used as platform for producing novel Fluvirucin B1 analogues using an active-site mutagenesis strategy. In the absence of protein-protein interaction effects, substrate selectivity is the key factor determining proper intermediate flow in these engineered systems. The KS substrate selectivity of downstream enzymes has been planned to test the feasibility of the mutagenesis strategy and determine the role of substrate selectivity. The FluKSAT3 has been analyzed using this method demonstrating its ability to provide a simple visual readout for the KS substrate preferences.

These preliminary studies lay a good groundwork for further extensive KS substrate analysis. The FluKSAT3 analysis concept will be extended to all the remaining four KSs starting with analysis of their native substrate preferences and extending it into non-native substrates with variable β-functionalities. The information gathered about substrate preferences of all the KSs will map out the possibilities in terms of engineering novel Fluvirucin analogues. The availability of this critical information about KS preferences will help design of active-site mutagenesis strategies with increased confidence in the execution of this scheme. Rather than

embarking upon a strategy with no prior information about the enzyme selectivity, the vital information about KS substrate preferences will definitely improve the success rate of producing diversified and novel polyketide analogues of Fluvirucin and other known and new PKS systems for therapeutic applications.

Another interesting application of this method could be examining the impact of substrate binding pocket mutations within KS domains on substrate tolerance. To begin with, crystal structures of the KS binding-site pocket will be examined and combined with homology studies to identify some potential amino acids involve in key interactions with substrates. Systematic mutations of these potential residues in the binding pocket of the KS can be performed to identify actual residues responsible for substrate preferences displayed by the KS. A series of KS mutant didomains as well as full modules will be prepared and analyzed against various substrates by using fluorescence transfer assay. By means of a simple visual read-out, this fluorescence assay can easily pick out KS mutants that display altered substrate tolerance and in turn identify the residue that has undergone mutation/alteration. Overall, PKS engineering efforts often suffer from poor yields due to lack of information about enzyme preferences. By identifying key residues in KS that determine substrate tolerance, these residues can be manipulated to alter substrate tolerance as necessary and greatly help PKS engineering efforts by improving yields in new and re-engineered systems.

A great advantage of this assay is that due its flexibility, simple set-up with quick visual read-out, it can be easily adapted to a medium/high-throughput kind of application where any KS in question can be rapidly assessed against a large panel of natural and non-natural substrates. This incredible feature will undoubtedly make it a popular choice for use in performing polyketide engineering to make novel polyketide products. We expect that the successful application of this approach will provide researchers a reliable tool to overcome the

challenge posed by strict enzyme selectivity and pave way to an era of highly simplified and productive PKS engineering.

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